FUNCTION AND REGULATION OF HUMAN AND SOYBEAN HEAT SHOCK TRANSCRIPTION FACTORS EXPRESSED IN YEAST AND HELA CELLS

By

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LIST OF VECTORS

Vectors page

LIST OF ABBREVIATIONS

aa.....Amino acid

AD1 (aa 401-420).....The first activation domain of hHSF1

AD2 (aa 430-529).....The second activation domain of hHSF1

β-gal.....β-galactosidase

bp.....base pair

CE1, 2.....Two short conserved elements of yHSF

CMV.....Cytomegalovirus

CTA1 (aa 422-529).....C-terminal activator 1 of hHSF1

CTA2 (aa 397-536).....C-terminal activator 2 of hHSF2

CTA1-Plus (aa 382-529).....CTA1 plus HR-C of hHSF1

CTD.....C-terminal domain of pol II

DBD.....DNA binding domain

dHSF.....*Drosophila* HSF

DMP.....Dimethylpimelimidate

ECL.....Enhanced chemiluminescense

5-FOA.....5-fluoroorotic acid

GAGA.....*Drosophila* transcription factor

GAL4.....Galactose regulated transactivator from yeast

GAL4-DBD.....GAL4 DNA binding domain

GmHSFs.....HSFs from *Glysine max* L. cv. Williams

GST.....Glutathione S-transferase

GTF.....General transcription factor

hGH.....Human growth hormone

hHSF.....Human HSF

HR-A.....Hydrophobic heptapeptide repeat A

HR-B.....Hydrophobic heptapeptide repeat B

HR-C (aa 382-422).....Hydrophobic heptapeptide repeat C

hr.....hour

hs.....Heat shock

HSE.....Heat shock element

HSFs.....Heat shock factors

Hsp.....Heat shock protein

klHSF.....*K. lactis* HSF

luc.....Luciferase

LpHSF.....HSF of tomato (*Lycopericon peruvianum*)

mHSF.....Mouse HSF

NLS.....Nuclear localization signal

NR.....Negative regulation domain (aa 212-310) of hHSF1

OD.....Oligomerization domain

PCs.....Positive cofactors

PIC.....Pre-initiation complex

pol II.....RNA polymerase II

pXGH5.....Human growth hormone vector with mMT-1 promoter

scHSF.....*S. cerevisiae* HSF

TAFs.....TBP-associated factors

TBP.....TATA binding protein

TFIIA.....Transcription factor A for pol II

TFIIB.....Transcription factor B for pol II

TFIID.....Transcription factor D for pol II

VP16.....Herpes viral protein 16

yHSF.....Yeast HSF

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Activities of human and soybean heat shock transcription factors (HSFs) were characterized in HeLa and yeast cells. Transcriptional activation domains were mapped to the C-termini of human HSF1 and HSF2 using GAL4 DNA binding domain fusions. The most C-terminal activation domain of human HSF1 was composed of multiple subdomains that seem to function synergistically. No direct evidence of transcriptional activity was observed for GmHSF34 and GmHSF5 from soybean. A general conservation in mechanism exists between humans, plants and yeast, since both human HSFs and soybean GmHSF5 were able to substitute in yeast for the endogenous HSF. Similar activity patterns for human HSFs were observed under heat shock and basal conditions in HeLa and yeast, except for

slight differences in the kinetics of the heat shock response.

Protein-protein interactions between HSF1 and general transcription factors (TFIIB, TBP, TAF32, TAF55 and PC4) were characterized in order to identify potential targets of contact in the transcriptional preinitiation complex. These contacts represent one of the final steps in heat stress induced transcription of heat shock genes. TATA binding protein (TBP) and transcription factor IIB (TFIIB) were identified as major targets for HSF1 transcriptional activation domains based on *in vitro* interaction assays. Co-immunoprecipitation of transiently expressed proteins in HeLa cells and squelch-rescue assays confirmed predictions based on *in vitro* results that interactions between HSF1 activation domains and TBP and TFIIB can occur *in vivo*. A negative regulatory region (NR) of HSF1 was shown to bind TFIID in nuclear extracts through contacts that probably involve TATA associated proteins (TAFs). These results suggest a model for transcriptional regulation by HSF1 that involves a shift in the equilibrium between formation of dysfunctional TFIID complexes with the NR and functionally competent complexes with the C-terminal activation domains.

INTRODUCTION

In most organisms a sudden elevation in temperature of approximately 10°C results in a major shift in transcriptional expression. Expression of most normal genes is attenuated to varying degrees and a subset of genes known as heat shock genes are activated (Ashburner and Bonner, 1979; Craig and Gross, 1991). In soybean as many as 19,000 copies of transcripts per cell encoding low molecular weight heat shock proteins (Hsps) accumulate over a 2 hour (hr) period (Schöffl and Key, 1982). The protein that mediates this massive induction of transcription is the heat shock transcription factor (HSF).

In eukaryotes other than yeast, activation of HSF is thought to involve a conformational change of pre-existing HSF accompanied by trimerization of identical subunits converting the protein from a non-DNA binding form to one that binds the heat shock consensus element (HSE) located in the 5'-flanking region of heat shock genes (Westwood et al., 1991). The HSE is required for heat induced activation of the promoter and is comprised of at least three tandem repeats of a 5 base pair (bp) sequence (5'-nGAAn-3') arranged in alternating orientations (Amin et al., 1988; Barros et al., 1992; Pelham, 1982; Xiao et al., 1991). Maintenance of HSF in a repressed state under non-heat shock conditions (basal repression) is thought to involve interactions between

HSF and cellular factors such as Hsp70 (Kim et al., 1995; Rabindran et al., 1994), and/or Hsp90 (Nadeau et al., 1993).

More than twenty HSFs from different species including yeast, *Drosophila*, mouse, HeLa cell, tomato, soybean, maize and *Arabidopsis* have been cloned as cDNAs and sequenced (Czarnecka-Verner et al., 1995; Gagliardi et al., 1995; Rabindran et al., 1991; Sarge et al., 1993; Sarge et al., 1991; Scharf et al., 1990; Schuetz et al., 1991) (M. D. Barros *et al.*, unpublished). In animals there exist more than three major classes of HSFs with distinct lineages, while in plants two major classes with sub-groups in each class are known (Czarnecka-Verner et al., 1995; Nover et al., 1996). Class A in plants is transcriptionally active, however, class B is inactive except LpHSF24 (Czarnecka-Verner, unpublished observation). Across kingdoms each class is distinct in amino acid sequence structure indicating a complexity in lineage and, perhaps, specialization in HSFs among eukaryotes.

The various HSFs show relatively little overall amino acid identity, but are considerably conserved in the DNA binding (DBD) and oligomerization domains (OD). The DBD comprises a three helix cluster typical of helix-turn-helix proteins, and the OD consists of a series of 4, 3-$(abcdefg)_n$ pattern hydrophobic heptad repeats (HR-A and HR-B) forming a coiled-coil during trimerization (Sorger and Nelson, 1989). Activation domains of the *Saccharomyces cerevisiae* HSF (scHSF) are located in both the N-terminal and C-terminal

regions of the protein (Chen et al., 1993; Jakobsen and Pelham, 1991; Nieto-Sotelo et al., 1990). The C-terminal activation domain is the strongest and best characterized and appears to be required for the transient heat shock response as well as sustained growth at high temperatures (Chen et al., 1993; Nieto-Sotelo et al., 1990). In tomato three HSFs have been cloned and a tryptophan motif (Trp-repeat) located C-terminal to the OD has been associated with transcriptional activity in transient assays using tobacco protoplasts (Treuter et al., 1993). The activation domains of mouse HSF1 (aa 425-471) and HSF2 (aa 473-517) are C-terminal to the third hydrophobic repeat (C-terminus) and possess an overall negative charge (Shi et al., 1995). The activation domain of *Drosophila* HSF is also located in the C-terminus and rich in acidic and hydrophobic amino acid residues (Wisniewski et al., 1996).

The activation and repression domains of hHSF1 have become some of the best characterized in higher eukaryotes (Green et al., 1995; Newton et al., 1996). The activation domains (ADs) of hHSF1 consist of two direct tandem domains at the C-terminus. Activation domain 1 (AD1) is rich in acidic and hydrophobic residues and is predicted to form an α-helix similar to a typical acidic activator. However, AD2 is a negative charge-, proline- and serine-rich activator without any predictable secondary structure. The activation and regulation domains of hHSF2 remain largely

uncharacterized. It is also unknown whether the regulation mechanism of HSFs across kingdoms is conserved.

The initial objective of this research is to characterize expression of soybean HSFs in yeast cells to evaluate the degree of conservation in mechanisms of basal repression and activation between plants and fungi. Similar experiments were conducted in parallel with human HSFs in yeast and HeLa cells as a form of positive control using well characterized HSFs, since the soybean HSFs were recently cloned and not characterized. For functional domain mapping of HSFs, the GAL4 DNA binding domain (GAL4-DBD) was fused to the N-terminus of the HSFs and the reporter genes for monitoring HSF activity were the GAL promoter joined to *lacZ* (Chevray and Nathans, 1992) for the yeast assay system and the GAL4-binding-site-TATA promoter fused with the luciferase (luc) gene for the HeLa assay system. This strategy eliminated background activity from endogenous HSFs and allowed deletion of the natural DBD and nuclear localization signal (NLS), since these were provided by the GAL4-DBD. Although use of GAL4-DBD fusions provided advantages for functional domain mapping, it was not suitable for addressing questions regarding basal repression of HSF activity and overall compatibility of the plant and human HSFs with yeast. For this purpose, substitutions of human and soybean HSFs in yeast were employed to monitor the heat shock response of heterologous HSFs in yeast using an HSE containing promoter

to drive the reporter gene. The endogenous plasmid-borne scHSF was eliminated by growth on 5-FOA plates.

After functional domain analysis of the HSFs in both heterologous and homologous systems under heat shock and non-heat shock conditions, a natural extension of these studies was to more directly address questions of mechanism in the activation of HSFs by identifying targets of contact for HSFs in the transcriptional preinitiation complex (PIC).

Transcription has at least three steps: initiation, elongation and termination. Transcriptional initiation by RNA pol II seems to require the ordered assembly of general transcription factors (TFIID, TFIIB, TFIIA, TFIIE, TFIIF, TFIIG/TFIIJ, TFIIH/BTF2 and pol II) into the PIC (Buratowski, 1994; Conaway and Conaway, 1993; Roeder, 1991; Zawel and Reinberg, 1993). It is believed that the interaction of transcriptional activators with any component of the PIC is required for transcriptional activation. In some cases, the binding affinities of transcriptional activators to some protein members of the PIC are often strongly correlated with transcriptional activating potentials of activators (Wu et al., 1996). Therefore, the first step in studying the mechanism of activation by transcriptional activators is the identification of potential targets proteins within the PIC followed by an evaluation of the functional relevance of the interactions *in vivo*. As the second major objective of this dissertation research, several kinds of strategies were employed to identify functional targets of hHSF1. First,

various *in vivo* and *in vitro* protein-protein interaction techniques were used to test the affinity of hHSF1 with general transcription factors and cofactors including hTBP, hTFIIB, PC4, hTAF32 and hTAF55. Assays involving immobilized GST-fusion proteins (GST-pull-down assays) were used to detect the protein-protein interactions *in vitro* allowing precise mapping of the interaction sites. Since interactions observed in the *in vitro* assay may be due to the relatively high concentrations of proteins used and the high sensitivity of detection methods, not all interactions observed *in vitro* may be biologically significant in living cells. To reduce the enforced nature of *in vitro* assay, co-immunoprecipitation of co-expressed proteins was adopted to further test the validity of interactions observed from GST-pull-down assays. Proof that the observed interactions are specific and biologically relevant finally requires a functional assay *in vivo*. An assay based on the squelching of activator-mediated transcription (squelch-rescue assay) was used to confirm that potential interaction can occur *in vivo* and have functional roles in transcriptional activation. All of the strategies discussed above depend on the availability of the proteins known to be components of the PIC, and are based on the assumption that these are the only possible targets. To compensate this drawback, the yeast two-hybrid system and far-western approaches were used to select possible unknown target proteins. Through a series of tests, two general

transcription factors emerged as likely targets of contact for hHSF1 C-terminal activation domains, hTBP and hTFIIB.

LITERATURE REVIEW

Heat Shock Response and Heat Shock Proteins

Living cells can sense adverse environmental stimuli and display a rapid molecular response. Under heat shock conditions, the expression of most genes is greatly attenuated and a relatively small subset of genes are highly induced at the transcriptional level. These heat inducible genes encode several classes of molecular chaperones known as heat shock proteins (Hsp). The spectrum of Hsps synthesized in different organisms show notable similarities. Hsps are highly conserved between diverse organisms are classified into related groups according to their average molecular weights: Hsp100, Hsp90, Hsp70, Hsp 60, and small Hsps (14 to 27 kDa). The Hsp70 family contains the most highly conserved proteins (Roberts and Key, 1991) and its members have diverse functions (Pelham, 1990) including renaturation of denatured and aggregated proteins (Pelham, 1986), folding and assembly of nascent proteins (Beckmann et al., 1990; Craig, 1993), and facilitation of protein translocation (Deshaies et al., 1988). The Hsp60 family is localized to the mitochondria and is also involved in proper folding and assembly of proteins (Craig, 1993; Ellis and van der Vies, 1991; Nadeau et al., 1993). The Hsp90 family, often in complexes with Hsp70

and/or Hsp60, function as regulators by interacting with various cellular proteins such as hormone receptors (Renoir et al., 1986), kinases (Oppermann et al., 1981), actin and tubulin (Mager and Kruijff, 1995). Members of the Hsp100 family are involved the acquisition of stress tolerance (Parsell et al., 1994). Small Hsp are thought to protect cytoplasmic proteins from forming irreversible aggregates at high temperature (Mager and Kruijff, 1995; Mansfield and Key, 1987). In plants this group proteins are highly expressed upon heat stress (Helm et al., 1990).

Function, Structure and Regulation of Heat Shock Transcription Factors

Function of HSFs and DNA Binding

The analysis of inducible gene expression has provided many insights into the molecular mechanisms of transcriptional control. Activation of heat shock (hs) genes in response to elevated temperature and other environmental stresses has been studied extensively as a paradigm for inducible gene expression (Ashburner and Bonner, 1979; Craig and Gross, 1991; Schöffl and Key, 1982). These studies have demonstrated that induction of hs genes in eukaryotes is mediated by the activation of preexisting heat shock transcription factors (HSFs) which in turn, bind to specific cis-elements within heat inducible promoters. These upstream heat shock elements (HSEs) are comprised of three to nine

repeats of a 5-bp unit, nGAAn, arranged in inverted orientation (Amin et al., 1988; Barros et al., 1992; Pelham, 1982; Xiao et al., 1991). Each of these 5-bp sites is able to bind a subunit of the HSF oligomer. When higher eukaryotes are exposed to hs, non-DNA-binding HSFs (monomers) are converted into active trimers (Westwood et al., 1991). Trimeric HSF binding to HSE is highly cooperative (Topol et al., 1985; Xiao et al., 1991) so that promoters containing multiple HSEs serve as platforms for high stable HSF-DNA complexes (Xiao et al., 1991). Exploitation of cooperativity in the numbers, and arrangement of the 5-bp units and the degree of match with the consensus may provide a mechanism for the the differential binding of HSFs to hs promoters resulting in a selective heat shock response (Lindquist, 1980). This theme of differential binding of HSF for selective gene expression is further expanded by the observation that mHSF1 and mHSF2 show large differences in the degree that cooperativity influence DNA binding (Kroeger et al., 1993). This may be one of the reasons why the highly cooperative HSF1 is involved in the heat shock response and HSF2 which does not show cooperativity plays a role in developmentally regulated HSF expression.

Characteristics of the DNA Binding Domain

DNA sequence and deduced aa sequence of HSFs indicate that HSFs are not highly conserved in overall sequence; however, all HSFs contain isolated domains which have been

highly conserved during evolution. The functional domains identified within HSFs are the DNA binding domain (DBD), the oligomerization domain (OD) and the transcriptional activation domain (AD) (Nover et al., 1996).

The phenomenon of highly conserved HSEs located upstream of all hs genes strongly suggests that all HSFs share a very similar structure in terms of the DBD, and function in a similar manner. Amino acid sequences located near the N-terminus of HSFs are the most highly conserved within the protein and constitute the DBD which consists of four antiparallel β-sheets and three-helix clusters typical of a helix-turn motif (Damberger et al., 1995; Harrison et al., 1994). The DBD is required for specific interaction with the HSE and may also function in the repression of transcriptional activity during normal growth, at least in yeast. For example, a single point mutation (M232V) in the scHSF (HSF from *S. cerevisiae*) DBD (Wiederrecht et al., 1988) results in constitutive transcriptional activity (Bonner et al., 1992). The ancient origins of the DBD are reflected in the conservation of 26 aa with the putative recognition helix of bacterial sigma factors. The motif is conserved in dHSF (HSF from Drosophila), scHSF, klHSF (HSF from K. lactis), LpHSF24 (HSF from tomato) and bacterial sigma 32 (hs sigma) and sigma 70 factors (Clos et al., 1990; Sarge et al., 1991). Sigma 32 is required for transient activation and sigma 24 for permanent activation under severe hs conditions in *E. coli*.

Plant DBDs are unique in that all plant HSFs (soybean, tomato, *Arabidopsis* and maize) seem to have a deletion of 11 aa residues between β3- and β4-strands meaning these two strands are connected by a turn of 4 aa residues instead of the much larger loop present in animal and yeast HSFs (Nover et al., 1996). In addition, parsimony analysis of DBDs suggest that plant HSFs can be classified into two major groups, A and B, containing multiple members with representatives in every species (Czarnecka-Verner et al., 1995; Nover et al., 1996). There is no apparent correlation between the lineage groups delineated in plants and functional classes present in mammalian and avian species, or close relatedness to the HSFs of yeast (Nover et al., 1996).

Properties of the Oligomerization Domain

The OD, consisting of two blocks of hydrophobic heptad repeats (HR-A and HR-B), is involved directly in protein-protein interactions in the formation of the trimeric form of HSF which is then able to efficiently bind DNA (Sarge et al., 1993; Westwood and Wu, 1993). In addition to its role in trimerization, the OD, along with the HR-C in the C-terminal region of HSFs, is required for sequestration of HSFs in the cytosol under non-heat shock conditions; deletion of either of these two regions results in constitutive nuclear localization of hHSF2 (Sheldon and Kingston, 1993). The OD is located C-terminal to the DBD and shows a high degree of conservation in its overall pattern of hydrophobic residues

(Peteranderl and Nelson, 1992; Sorger and Nelson, 1989), but little conservation in the specific aa sequence varying from 41% between yeast and human HSF1, to 79% between HSFs and Drosophila HSF (Rabindran et al., 1991). The hydrophobic region takes the form of a heptad repeat characterized by the occurrence of a bulky hydrophobic aa, commonly Leu, Ils, or Val, in every first position ("*a*" position) and at every fourth position ("*d*" position) within the seven residue repeat (*abcdefg*) typical of bZip proteins. These amino acids form a hydrophobic surface that provides the region of contact between paired helices (Sorger and Nelson, 1989). Although hydrophobic interactions between *a* and *d* positions in different strands are the major stabilizing force in triple-stranded coiled-coils, the specificity of trimerization is thought to be conferred by charged residues at the *e* and *g* positions (Alber, 1992). If the inactive form of HSF is maintained in a metastable state by various molecular forces, for instance, by a combination of hydrophobic, charged, and polar interactions, then the perturbation of a subset of these forces by any one inducer of the stress response could be sufficient to initiate the trimerization.

The ODs of plant HSFs show a similar overall pattern of heptad repeats as that found in other eukaryotic organisms; however, plant HR-As are shorter, frequently have a hydrophobic amino acid other than leucine at the *d* position of the fourth and fifth heptads, and often have zipper

destabilizing residues at the *a* position. All these differences are predicted to make the interactions in plant HR-A region weaker and the trimer of plant HSFs less stable (Czarnecka-Verner et al., 1995).

The ODs of plant HSFs appear to have two types: type I including animal and yeast HSFs in addition to AtHSF1, LpHSF8, LpHSF30 as well as GmHSF21; type II unique to plants, consisting of LpHSF24, GmHSF5, GmHSF29, GmHSF31 and GmHSF34 (Czarnecka-Verner et al., 1995). The two classes of ODs correspond exactly with the plant HSF groups A and B derived from parsimony analysis of the DBD (Group A = type I, Group B = type II). The type I OD is demonstrated by the inclusion of a glutamine-rich region between HR-A and HR-B, but the HR-A and HR-B of type II are adjacent. The other difference between the two types is the spacing between the OD and the DBD. In type I, the separation is only 12 to 26 aa, but in type II the spacing is much larger, 50 to 74 aa (Czarnecka-Verner et al., 1995). The functional and evolutionary significance of these two major classes of plant HSFs is still unclear; however, a preponderance of the Group B HSFs (type II OD) have no transcriptional activity in transient assays (Czarnecka-Verner et al, unpublished).

Attributes of Activation Domain

Activation domains (ADs) are frequently located at the C-termini of HSFs (Hoj and Jakobsen, 1994; Nakai and Morimoto, 1993; Newton et al., 1996; Nieto-Sotelo et al.,

1990; Rabindran et al., 1993; Shi et al., 1995; Zuo et al., 1995). Yeast HSF (yHSF) contains at least two domains that are capable of functioning as transcriptional activators. The two domains located in the N-terminus (CE1) and C-terminus (C-terminal domain) of the HSF, can provide either constitutive, or unregulated transcriptional activity, respectively. Transcriptional activity of the C-terminal domain is much more stronger than that of CE1 (Chen et al., 1993; Jakobsen and Pelham, 1991; Nieto-Sotelo et al., 1990).

In both mHSF and dHSF the C-terminal activation domains show little amino sequence conservation and are rich in hydrophobic and acidic residues (Shi et al., 1995; Wisniewski et al., 1996). hHSF1 seems to have two separable tandem ADs (AD1 and AD2) at the C-terminus (Green et al., 1995; Newton et al., 1996). AD1 contains only 20 aa residues embedded in HR-C; AD2 is located in the extreme C-terminal region corresponding to the CTA1 (C-terminal activator 1) originally defined in this dissertation research. The natural fusion of the HR-C and CTA1 forms the CTA1-Plus domain encompassing all of AD1 and AD2. AD1/HR-C and AD2/CTA1 show distinct differences in terms of aa composition and predicted secondary structure. AD1/HR-C is rich in acidic and hydrophobic residues and predicted to form an amphipathic α-helix similar to those typical of acidic activators such as GAL4, VP16. Although AD2/CTA1 is acidic (13% negative) and rich in hydrophobic aa (13% Leu) similar to AD1/HR-C, it also contains high percentages of proline (18%), serine (24%) and

glycine (8%), and seems unable to form either an α-helix or β-strand. A comparison of aa compositions of typical proline-rich activators with AD2/CTA1 is presented in table 3. Based on the aa composition and the low probability of forming secondary structures, it seems more appropriate to define the AD2/CTA1 as a proline-rich activator instead of acidic activator. Therefore, hHSF1 seems to contain two distinct activation domains at the C-terminus, one acidic and the other proline-rich. The close apposition of the AD1/HR-C and AD2/CTA1 makes it likely that these two activation domains function synergistically to activate transcription as shown for TFE3 activator domains (Artandi et al., 1995).

In tomato three HSFs have been cloned and a trp-repeat located C-terminal to the OD has been associated with transcriptional activity in transient assays using tobacco protoplasts (Treuter et al., 1993). Mutagenesis analysis of the repeat indicated that trp is not important for activator activities *per se*. However, the short peptide motifs rich in aromatic, large hydrophobic and acidic aa residues (AHA motif) seems related with transcription (Nover et al., 1996).

Role of Masking in Yeast HSFs

In the masking model of HSF regulation, it is proposed that the N-terminal masking domain interacts with the C-terminal transcriptional activator to block its function. The critical experiments that support this hypothesis are a series of functional domain mapping and domain swapping

studies of yHSF indicating the importance of global protein structure in the regulation of transcriptional activity (Bonner et al., 1992; Jakobsen and Pelham, 1991; Nieto-Sotelo et al., 1990; Sorger, 1990). The most crucial experimental result which severed as the original basis for proposing that HSF activation domains were masked under basal conditions was the gain of constitutive activity that occurred when the N-terminal 146 aa region of yHSF was deleted (Sorger, 1990). An HSF monomer was envisioned to be essentially folded in half in such a way that the activation domains are inaccessible and not functional. Another variation of the masking model requires that the masking of the AD is the result of interactions with a non-HSF protein (intermolecular masking). In this scheme, masking is alleviated by dissociation of the second protein resulting from a conformational change in the HSF. Regions of yHSF that are potentially involved in masking are the two conserved elements (CE1 and CE2), the DBD and the OD (Bonner et al., 1992; Jakobsen and Pelham, 1991; Nieto-Sotelo et al., 1990; Sorger, 1990). Since mutations within the OD cause relief of repression of HSF activity under non-heat shock conditions, repression of the AD seems to require the normal oligomerization state of the HSF molecules (Chen et al., 1993). Mutations within the DBD can also initiate the loss of repression of HSF activity (Bonner et al., 1992). This may indicate that global structure is important for masking and complete repression of transcriptional activity. CE2 is

crucial for masking and repression since its deletion causes yHSF to be constitutively active (Jakobsen and Pelham, 1991), presumably through releasing the C-terminus and permitting it to interact with the transcription initiation complex. The first 8 aa residues of CE2 are believed to bind either to the structural core of HSF, or to another polypeptide to maintain the AD in an inactive conformation (Jakobsen and Pelham, 1991). CE2 seems to function by stabilization of the hypothetically folded structure of the repressed form of yHSF. CE1 also seems to contribute to repression, but is subsidiary to CE2 in its role (Jakobsen and Pelham, 1991).

The DBD is also essential for repression of yHSF. Conversion of Met 232 to Val in the DBD has a very dramatic effect, releasing repression almost completely (Bonner et al., 1992). This suggests that Met 232 is intimately involved in the maintenance of the repressed (masked) state of yHSF, although the exact role of this residue is not clear. The location of Met 232 within the DBD indicates that this region may be bifunctional, involved in both protein-DNA interactions and protein-protein interactions.

The intramolecular masking model of HSF seems unsuitable to mammalian HSFs. Functional domain mapping and domain swapping experiments from mHSF1 and hHSF1 indicated that global protein structure is not required for basal repression. The DBD and HR-A deletions of mHSF1 had no effects on basal repression, or heat shock induction of transcriptional activity (Shi et al., 1995), and complete

deletion of the DBD and the OD (HR-A and HR-B) of hHSF1 had no effect on heat inducibility of AD1 (Newton et al., 1996). Furthermore, the entire mechanism of heat inducibility seems to reside in the negative regulation domain (NR) which encompassed only 90 aa. The VP16 activator was rendered heat inducible after joining a GAL4-DBD-NR fusion to its N-terminus (Newton et al., 1996). The precise mechanism whereby the NR confers heat inducible regulation to AD1 and the heterologous VP16 is still an intriguing question. It may be that a modified version of the intermolecular masking model still applies, but only involves the NR and a C-terminally located activation domain(s).

Trimerization

Trimerization is essential for high affinity binding of HSFs to HSEs (Lis and Wu, 1993; Sorger and Nelson, 1989; Westwood et al., 1991; Westwood and Wu, 1993), since both the monomeric and dimeric forms of HSF have low affinity for HSEs (Sarge et al., 1993). HSF binding in eukaryotes other than yeast is regulated by a change in the oligomerization state upon hs. HSF binding to HSEs is highly synergistic at two distinct levels: between subunits of the HSF trimer, and between trimers (Xiao et al., 1991). The binding of one dHSF trimer to an HSE with six nGAAn repeats enhances the binding of a second trimer by over 2,000-fold through protein interactions between the DBDs (or with the DBD plus the HR-A of the OD) (Wyman et al., 1995; Xiao et al., 1991). This

cooperativity is particularly important for the high-magnitude and promptness of the response. Why is HSF trimeric? In addition to structural requirements for DNA binding, a second reason may be that control of oligomerization plays a role in the regulation of the hs response via interaction with Hsp70 (Clos et al., 1990).

Regulation of HSFs by Cellular Proteins

Posttranslational modifications including phosphorylation may have the potential to affect the multimeric state of HSFs. Purified HSFs can be activated *in vitro* by heat (Larson et al., 1995) which suggests that the preexistent, inactive form of HSFs can assume the active conformation without biochemical modification of protein structure. Current thinking on HSF regulation suggests that a complex of molecular chaperones (Hsps) interact with HSF in the basal state to achieve masking and cytosolic localization. Maintenance of the inert state seems to occur through association with either constitutive (p72/hsc70) or inducible (Hsp70) members of the Hsp70 family (Kim et al., 1995; Mosser et al., 1993; Nunes and Calderwood, 1995). However, the interaction between heat shock factors and Hsp70 alone may not be sufficient to mask the activity of HSFs (Rabindran et al., 1994). Since Hsp90 has also been shown to associate with HSF by coimmunoprecipitation studies (Nadeau et al., 1993), it may have a role in the regulation of HSF activity as well. General support for models of HSF

regulation involving inhibitory molecules is seen in the following two observations. First, hHSF1 is activated below its normal temperature when expressed in heterologous cells such as *Drosophila*, tobacco protoplasts, or frog oocytes which implies that additional cellular factors are involved in regulation (Clos et al., 1993; Treuter et al., 1993). Second, recombinant HSFs from HeLa, *Drosophila* and tomato exhibit constitutive HSE binding activity when expressed in *E.coli* (Clos et al., 1990; Rabindran et al., 1991; Scharf et al., 1990; Schuetz et al., 1991), presumably due to the lacks of regulatory proteins that would normally repress its activity under non-hs conditions.

Role of Hsp70

There are several studies suggesting a regulatory connection between Hsp70 levels and the transcription of hs genes in eukaryotes (Craig and Gross, 1991; DiDomenico et al., 1982; Kim et al., 1995; Morimoto et al., 1990; Mosser et al., 1993; Nunes and Calderwood, 1995; Stone and Craig, 1990). Exposure of HeLa cells to hs results in transient activation of HSF; its DNA-binding activity increases rapidly, plateaus and attenuates, during which the intracellular levels of Hsp70 increase (Abravaya et al., 1991). Injection of denatured protein into unstressed *Xenopus* oocytes stimulates the activation of HSFs (Mifflin and Cohen, 1994a) and co-injection with Hsp70 can antagonize this induction (Mifflin and Cohen, 1994b). Several studies

strongly suggest that Hsp regulates its own synthesis by directly interacting with HSF, although not supported by hydrodynamic studies (Sistonen et al., 1994; Westwood and Wu, 1993). Hsp70 has been shown to be able to bind activated HSFs *in vivo* using non-denaturing gel electrophoresis and co-immunoprecipitation techniques (Abravaya et al., 1992; Baler et al., 1992; Rabindran et al., 1994). It has also been shown *in vitro* that Hsp70 can block the activation of HSFs from a non-DNA binding state to a DNA-binding form (Abravaya et al., 1992; Clos et al., 1990; Sorger, 1991). This blocking effect is abolished by the addition of ATP (Abravaya et al., 1992).

It has been proposed that Hsp70 acts the cellular temperature sensor (Craig and Gross, 1991). The pattern of Hsp70 expression suggests that Hsp70 can interact with HSFs at normal temperatures and repress HSF activation. Damaged proteins resulting from partial thermal denaturation compete for Hsp70, thus depleting the pool of Hsp70 and allowing HSFs to escape basal repression (Ananthan et al., 1986; Baler et al., 1992). Repression will be restored once the ratio of denatured protein substrates to Hsp70 has been returned to normal, either by the production of sufficient Hsp70, or by removal, or refolding of the substrates. Continuous exposure of yeast to high temperature stress reveals that an increase in the severity of the heat stress eventually abolishes the attenuation phase of HSF activation and alters the pattern of hs gene expression from transient to sustained (Chen et al.,

1993; Sorger, 1990). One interpretation of this phenomenon is that during prolonged hs, the continuous demand on Hsp70 for binding to damaged proteins depletes the pool of Hsp70. Hence, the negative regulation of Hsp70 is removed and the activity of stress-inducible HSFs remains constant without an attenuation phase. However, this view fails to explain why HSF is not eventually attenuated by the continuous accumulation of Hsp70. An alternative hypothesis is that severe hs decreases the Hsp70 affinity to HSFs dramatically, resulting in no Hsp70 binding to HSFs, even when Hsp70 is present. The affinity change is postulated to result from conformational changes of HSFs or Hsp70, or be conferred by perturbation of ATP levels (Benjamin et al., 1992).

Phosphorylation of HSFs

There is a strong correlation between HSF activation and HSF phosphorylation (Craig and Gross, 1991; Sorger, 1990; Sorger, 1991; Sorger and Pelham, 1988), but the role of HSF phosphorylation has remained elusive. hHSF1 is hyperphosphorylated during hs, but this phosphorylation is not required for either oligomerization or DNA binding (Larson et al., 1988; Larson et al., 1995). The transcriptional activation of hHSF1 also has no phosphorylation requirement (Newton et al., 1996). Removal of all possible phosphorylation sites in the AD1 of hHSF1 (Newton et al., 1996), as well as in the CE2 of scHSF (Jakobsen and Pelham, 1991), did not affect transcription.

In yeast, hs-induced phosphorylation is not necessary for the activation, but is required for HSF deactivation (Hoj and Jakobsen, 1994). The lack of a requirement for phosphorylation of the ADs is consistent with experiments where the hs response remained normal after replacement of the AD1 and AD2 of hHSF1 with the VP16 activator (Newton et al., 1996). In addition, structural changes of HSFs can be observed in the absence of phosphorylation. *In vitro*, hs can convert the purified human and mouse HSFs from the non-DNA binding form into an active DNA-binding form (Goodson and Sarge, 1995; Larson et al., 1995). This transition does not depend upon phosphorylation of HSFs, since a single aa mutation ($K_{298}A$) in the NR, which alone is sufficient for heat-inducibility, destroyed the heat activation (Newton et al., 1996). Taken together, it seems that phosphorylation of HSFs is not essential for transcriptional activation of HSFs.

The Steps of HSF Regulation

The finding of multiple HSFs in several animals and in plants (Nover et al., 1996), and of tissue-specific expression of different HSFs (Forenza et al., 1995; Goodson et al., 1995), raises the possibility that HSFs may be specialized for different stresses, or different functions in development (Sistonen et al., 1994; Treuter et al., 1993), and that they may be regulated differentially. For HSFs primarily responsible for the hs response instead of developmental processes, induction of gene expression relies

on an activation of preexisting HSFs. This activation process is composed of at least three separable steps. The first is thought to involve unmasking of the OD and a nuclear localization signal (NLS) resulting in trimerization and nuclear localization (Westwood et al., 1991; Westwood and Wu, 1993). The exposure of the OD for trimerization and the NLS for transportation into nuclei may be facilitated by removal of negative regulators such as Hsps from HSFs. The second step in activation involves cooperativity in binding of HSF to hs gene promoters (Xiao et al., 1991). This process may require auxiliary factors such as the GAGA factor in *Drosophila*, or AT-rich element binding factors in plants, for keeping the promoter in an "open configuration" (Czarnecka et al., 1992; Shopland et al., 1995). The final activation step occurs after DNA binding by the HSF trimer. Binding to promoter DNA is not sufficient for transcriptional activation as shown by the lack of transcriptional activity after treatment with salicylic acid or other anti-inflammatory agents even though HSF1 was shown to be localized in the nucleus and bound to the promoter (Jurivich et al., 1992). At present the mechanism for the final activation step remains largely speculative, but attention has been focused on the possibility of conformational change, since phosphorylation appears to have been ruled out.

The discussion above reviewed the regulation of HSFs activity without consideration of the large question regarding the mechanisms of transcriptional activation in

general. At present, these is very little known regarding HSF interactions with other components of the transcriptional apparatus. General pathways of transcriptional activation and possible mechanisms of HSF-mediated transcriptional activation will be discussed in next section of the Literature Review.

Mechanism of Transcriptional Activation

Transcriptional Activation and Promoter Structure

For RNA polymerase II promoters, transcription occurs at two levels, basal and activated. Basal transcription is directed by a core promoter and a minimum number of general transcription factors (GTFs). Activated transcription requires transcriptional activator proteins, activator-binding sites upstream of the core promoter, and all of the GTFs required for basal transcription, plus coactivators in some cases (Ge et al., 1994; Kretzschmar et al., 1994a; Parvin et al., 1994). A core promoter is sufficient for basal transcription, but not for activated, since it lacks sites for upstream activators to bind. Core-promoters are highly variable in DNA sequence, yet all contain one or two recognizable sequence elements, the TATA box and/or initiator element (Inr). Most core-promoters contain TATA boxes including the core-promoters of most plant hs genes (Czarnecka et al., 1992; Gurley et al., 1992). Other core promoters have an Inr but lack a TATA box (Struhl, 1989), as

is the case for a petunia Hsp70 gene (Winter et al., 1988). A small numbers of genes contain both elements, or neither (Emami et al., 1995). The requirement of a TATA box for basal and hs-inducible expression of most hs genes has been shown in plant and animals (Czarnecka et al., 1989; Williams and Morimoto, 1990). For TATA-less hs promoters, some other DNA element, for example, a Inr, must be present to substitute the role of TATA box (Winter et al., 1988).

The promoter structures upstream of core promoters may be complex depending on the particular gene. For example, in many plant hs gene promoters, the region upstream of the TATA box contains multiple AT-rich elements including AT-composite and AT proximal elements, and several HSEs (Barros et al., 1992; Czarnecka et al., 1992; Gurley et al., 1992). The upstream elements can be organized in clusters to form enhancers, or exist as individual DNA binding sites for specific transcriptional activators (Jones, 1994; Struhl, 1989). The modular nature of *cis* elements means that promoter expression can be dictated simply by grafting specific elements into existing promoters, or minimal promoters. This strategy is often employed in the design of reporter genes useful in the characterization of activator proteins (Bonner et al., 1992; Patel et al., 1995)

Models of Transcriptional Activation by Activators

Many activators have been shown to make physical contact with GTFs in a process that leads to transcriptional

activation. The recruitment of GTFs by activators is thought to be the key mechanism for activator-mediated transcriptional activation. Activators have been shown to recruit their targets to the promoter and thereby, enhance the assembly of the PIC (Barberis et al., 1995; Roberts et al., 1995). Stabilization of the PIC, in turn, increases the rate of initiation and/or the processivity of transcriptional elongation (Yankulov et al., 1994). When activators are bound far from the transcriptional initiation site, the distance problem of recruitment can be overcome by the flexibility of DNA in looping. For instance, distant HSE sites can be more than 1Kb upstream of the start site, and the separation between two HSEs can be several hundred bp apart (Wyman et al., 1995). In this case, HSF has been shown *in vitro* to bind to these distant sites and bring the two clusters of HSE together by DNA looping (Wyman et al., 1995). DNA looping also allows HSF to bring its targets into close proximity with the PIC, efficiently to recruit GTFs needed for transcriptional activation. This ability of DNA to loop provides opportunities for protein-protein interactions between activators, and between the activators and their targets to synergistically activate transcription.

During the recruitment process, conformational changes of the activators and their targets, as well as topological re-organization of the protein-promoter complex may occur (Hori and Carey, 1994; Oelgeschlager et al., 1996; Roberts and Green, 1994). There may be two consequences of

recruitment. If the conformational changes are productive, they may result in transcriptional activation; otherwise, nonproductive recruitment may lead to transcriptional repression. This hypothesis is supported by several experimental observations. One example involves the thyroid hormone receptor which can function both as an activator in the presence of thyroid hormones and as a repressor in the absence of the hormones. Unliganded thyroid hormone receptor alpha can recruit TBP in such a way that it inhibits the subsequent steps of PIC formation, and addition of thyroid hormone can turn this repression into activation (Fondell et al., 1995; Fondell et al., 1996). In this case, recruitment leads either to repression, or activation.

Synergism of Transcriptional Activation

In some genes, activators can bind to multiple binding sites and enhance transcription synergistically (Sauer et al., 1995a). The level of transcriptional activation by Bicoid (an activator from *Drosophila*) is correlated in a cooperative fashion with the number of the DNA binding sites (Sauer et al., 1995a). Some activators such as the activation domain of p65 NF-kB, VP16 and ZEBRA (a non-acidic activator) can interact with multiple targets to show a synergistic effect on transcriptional activation (Chi et al., 1995; Schmitz et al., 1995) presumably via cooperative recruitment of GTFs. In theory, HSFs have great potential to activate transcription in a synergistic manner. Since

typical hs promoters usually bind from six to twelve HSF monomer units (two to four trimers) (Czarnecka-Verner et al., 1994), cooperativity in target recruitment is very likely. The potential for synergism should be even greater if each HSF monomer has the capacity to recruit more than one type of target protein.

When different activators function on a same promoter, their combination may also produce synergistic effects on transcriptional activation (Chi et al., 1995; Sauer et al., 1995b) through interacting distinct components (such as several TAFs) of the PIC (Sauer et al., 1995a; Tjian and Maniatis, 1994). The two isolated activation domains (one is glutamine-rich; the other is alanine-rich) from Biocoid can synergistically stimulate transcription when they are fused to the hunchback (HB) DNA binding domain (Sauer et al., 1995a). A similar synergism has been demonstrated in the natural fusion of the two activation domains that exists in the Bicoid protein (Sauer et al., 1995a). The activator TFE3 contains two activation domains, one acidic and the other proline-rich, which also show cooperativity in the activation of transcription (Artandi et al., 1995). These examples illustrate the synergistic potential of activators consisting of multiple activation domains in transcriptional activation.

In the activation of hs genes, HSFs may utilize several strategies to activate transcription synergistically. First, hs gene promoters can provide a substrate for the binding of multiple HSFs (Czarnecka et al., 1992; Mager and Kruijff,

1995; Xiao et al., 1991). The synergism inherent in the binding of multiple HSF trimers is supported by the *in vitro* binding studies (Xiao et al., 1991), and the observations that a single HSE in animal, plants and yeast hs promoters is not sufficient for efficient transcription of hs genes (Dudler and Travers, 1984; Gurley and Key, 1991; Schöffl et al., 1989; Wei et al., 1986). Another possibility is that HSFs, such as hHSF1, may contain multiple activation domains contributing synergistically to transcriptional activation. Functional domain mapping of hHSF1 reported here will shed light on the number of targets and types of activation domains present in this HSF.

Transcriptional Initiation: A Key Step for the Regulation of Transcriptional Activation

One model for transcriptional initiation by pol II requires the ordered assembly of general transcription factors (TFIID, TFIIB, TFIIA, TFIIE, TFIIF, TFIIG/TFIIJ, TFIIH/BTF2 and pol II) into the PIC (Buratowski, 1994; Conaway and Conaway, 1993; Roeder, 1991; Zawel and Reinberg, 1993). The initial and rate-limiting step of this assembly process is the binding of TFIID to the TATA box (Klein and Struhl, 1994). The facilitated recruitment of TBP to nucleosomal DNA *in vivo* is shown to be critical in the regulation of eukaryotic gene expression (Imbalzano et al., 1994).

One simple model of transcriptional activation suggests that a few key components of the PIC, such as TBP/TFIID and/or TFIIB, are rate-limiting; the simple recruitment of these key factors by transcriptional activators can result in transcriptional activation (Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Klein and Struhl, 1994). For example, kinetic studies of TBP using specificity-altered TATA-promoter and mutated-TBP assay system (Klein and Struhl, 1994) suggest that the recruitment of TBP is rate-limiting *in vivo* (Klein and Struhl, 1994). The direct fusion of TBP with the LexA DNA binding domain results in the activation of transcription in the absence of transcriptional activators (Chatterjee and Struhl, 1995) indicating that the recruitment of TBP is the key step for transcriptional activation.

This recruitment model is not inconsistent with the RNA polymerase II holoenzyme idea (Barberis et al., 1995; Ossipow et al., 1995; Thompson and Young, 1995). According to this hypothesis, RNA polymerase II holoenzyme is a titanic protein complex including many GTFs and cofactors which are preformed before the complex binds to the promoter. The number of proteins thought to form the holoenzyme becomes larger as more proteins are continuously shown to be associated with the complex (Emili and Ingles, 1995; Ossipow et al., 1995). Activator-mediated recruitment of a single transcription factor, or even a mega-protein complex such as the holoenzyme, can facilitate transcriptional initiation and lead to transcriptional activation (Barberis et al., 1995).

Although other pathways to activation exist, such as keeping the chromatin template open (Paranjape et al., 1994), and stripping off the repressors (Kraus et al., 1994) for activator-mediated transcriptional activation; these routes seem to be limited to particular situations, not generalized mechanisms for a broad-spectrum of activator-mediated transcriptional activation. For HSF mediated transcriptional activation, it seems reasonable that at a minimum, HSF must have affinity for at least one GTF and recruit it to the PIC; however, this and other mechanisms of activation remain to be demonstrated.

Transcriptional Elongation: In Special Cases Expected to Achieve Transcriptional Activation

The complete assembly of a PIC on a given promoter is not always sufficient for productive and efficient transcription. In the case of some genes such as *HIV*, *Drosophila Hsp70* and human *c-myc*, the pivotal step of activation is elongation, a step where some activators may exert their influence (Bentley and Groudine, 1986; Cullen, 1993; Lis and Wu, 1993). Control of gene expression through the regulation of elongation has the potential advantage of achieving a quick response to activation because the PIC has been pre-assembled. Some activators such as Tat, VP16 and E1a, in addition to the facilitation of TATA-box binding by TFIID (Yankulov et al., 1994), can increase the processivity of transcriptional elongation (Shopland et al., 1995;

Yankulov et al., 1994). The degree of transcriptional processivity enhanced by acidic activators depends on the quantity, other than the quality, of activation modules, and the number of DNA binding sites (Blair et al., 1996). For example, GAL4 DNA binding domain fused with a trimer of acidic activation modules (AAM3) resulted in ~5-fold increase of processivity compared with that induced by a dimeric module fusion (Blair et al., 1996). Acidic activators such as VP16 and Rel A are individually sufficient for the stimulation of elongation (Blair et al., 1996), but in the *Drosophila* Hsp70 promoter, the stimulation of processivity by HSF requires the cooperation of the GAGA factor; dHSF alone has no effect on elongation (Shopland et al., 1995). Other activators, like the GAGA factor (Shopland et al., 1995), have a negative effect on processivity of transcription simply by stimulating the basal rate of initiation without affecting the elongation process. One model for gene activation suggests that in cells there are two kinds of transcriptional elongation complexes, nonprocessive and processive forms (Bentley, 1995; Yankulov et al., 1994). In the absence of activators, the nonprocessive form is dominant; however, in the presence of activators, processive complexes of elongation become dominant. The balance between the two forms is governed by the promoter elements, including the TATA-box and enhancers, and activators bound to these elements. This model may only be useful in special cases when applied to genes that exhibit a significant level of

aborted transcriptional elongation. Overall, it appears for most genes that the key regulatory mechanism for transcriptional activation involves the control of transcriptional initiation in which GTFs are recruited to the PIC through protein-protein interactions with activator proteins.

Targets of Activators in Transcriptional Activation

TBP and TFIID in Transcriptional Activation

According to the multistep assembly model, TBP/TFIID facilitates formation of the PIC by recognizing and binding the TATA box which serves as the nucleation point for building the PIC (Buratowski, 1994; Conaway and Conaway, 1993; Roeder, 1991; Zawel and Reinberg, 1993). TBP is a relatively small protein with a unique saddle shape and has been shown to bend DNA upon binding (Nikolov et al., 1992). TBP is believed to contain determinants for protein-protein interactions with pol II, certain TAFs and various sets of activators and repressors (Tang et al., 1996). TBP, a subunit of TFIID, was first cloned from yeast, and then from *Drosophila* and human cells (Hahn et al., 1989; Hoey et al., 1990; Hoffman et al., 1990). Although TBP was isolated as a single polypeptide from yeast, subsequent studies with *Drosophila* and human TBP showed that in these organisms TBP is tightly associated with multiple proteins called TAFs (TBP associated factors).

TFIID/TBP binding to the chromatin template is rate limiting in gene expression, and the kinetics of this process are subject to regulation by activators *in vivo* (Klages and Strubin, 1995; Klein and Struhl, 1994), and by SWI/SNFs (SWIs, yeast genes controlling gene the switching process; SNFs, yeast genes controlling sucrose nonfermentation) (Imbalzano et al., 1994). Activators bound to upstream regions of the promoter have been shown *in vivo* to increase recruitment of TBP to the TATA box (Klages and Strubin, 1995). When TBP was translationally fused with a DNA binding domain such as LexA, elevated levels of transcription occurred in the absence of activator protein (Chatterjee and Struhl, 1995). This artificial fusion connection mimics the interaction that normally occurs between a transcriptional activator and TBP or TFIID, and the recruitment of TBP/TFIID leading to transcriptional activation.

Since TBP is a small protein, it is surprisingly found that TBP can interact directly with numerous activators such as VP16, Tat, Tax-1, E1A, Zta, ICP4, v-Rel, c-Rel, NF-κB, p53, E2F-1, cFos, c-Jun, c-Myc, PU-1, Sp1 and SSAP (Defalco and Childs, 1996; Schmitz et al., 1995). In many cases, the strength of activator-TBP interactions correlates well with the ability of activators to stimulate transcription both *in vitro* and *in vivo* (Liu and Berk, 1995; Melcher and Johnson, 1995; Schmitz et al., 1995; Song et al., 1995; Wu et al., 1996). However, there are notable examples that seem to indicate little relevance between the ability of binding to

TBP and the strength of activation (Tansey and Herr, 1995). There are many possible explanations for this apparent discrepancy. First, the interactions detected between activators and TBP *in vitro* may be irrelevant in living cells due to the forced nature of interactions under nonphysiological conditions. Second, some bindings, which are very inefficient *in vitro*, may still be sufficient for transcriptional activation *in vivo*. A third possibility is that activators may be capable of activating transcription through multiple pathways *in vivo* where the redundancy in interactions may mask the effects of a single interaction on transcriptional activation. The multiple pathway scenario is especially attractive due to numerous examples of activator binding to TAFs.

A set of TBP mutants obtained from genetic screening by Lee and Struhl (Lee and Struhl, 1995) are defective in responding to acidic activators, but appear normal for constitutive transcription *in vivo*. This set of mutants shows loss of TATA box binding ability, but seem to interact normally with VP16 and TFIIB *in vitro*. These results imply that the concave surface of TBP not only recognizes and binds the TATA element, but certain undefined aspects of this interaction strongly influence activated versus basal expression. Similar mutants that disrupt DNA binding and are defective in activated, but not basal transcription have been characterized (Kim et al., 1994). These mutants have been shown to either disrupt VP16 binding, or prevent association

between TBP and TFIIA or TFIIB (Kim et al., 1994). From these results it seems likely that perturbations in the TATA binding surface may also effect a change in conformation affecting potential interactions with activators, TAFs and other components of the PIC. Other explanations have been offered to account for the behaviors of activation-specific mutants located on the DNA binding surface of TBP (Lee and Struhl, 1995). For example, activated transcription may require the TBP molecular to remain bound at TATA for a certain length of time, a property that might be affected by the mutant. Another possibility is that the TBP/DNA complex with the mutant TBP may not present a conformation that is responsive to activators even though activator binding itself is not effected.

Interestingly, TFIID can form homodimers through self-association of the TBP subunit *in vivo* (Taggart and Pugh, 1996) which is consistent with the dimeric crystal structure of TBP (Nikolov et al., 1992). The dimerized form of TFIID is inefficient in binding the TATA-box and is transcriptionally inactive. It has been suggested that an equilibrium exists between the monomeric DNA-binding form of TFIID and the dimeric non-DNA-binding TFIID that is subject to regulation by a battery of activators and repressors (Taggart and Pugh, 1996). This proposed mode of activation is highly speculative, since it seems to go beyond the simple recruitment model of TFIID to the promoter, suggesting that

regulatory proteins also directly influence the ratio of active versus inactive forms of TFIID.

TAFs of TFIID in Transcriptional Activation

In TAF-facilitated transcriptional activation, TAFs enhance activator-mediated recruitment of the TFIID complex to the promoter just by providing surfaces of contact for direct interactions between the TAFs and the activators. However, the complete mechanism by which TAFs function in the transcriptional activation is still unknown. Since some TAFs are able to interact with activators and GTFs such as TFIIA, TFIIB, TFIIF, pol II (Goodrich et al., 1993; Hisatake et al., 1995; Klemm et al., 1995; Ruppert and Tjian, 1995), the simultaneous interactions of TAFs with transcriptional activators and GTFs provide a way for cooperative binding to greatly facilitate the PIC formation and stability. In this role, TAFs function as a bridging factor between activators and GTFs.

It has been suggested that different TAFs may connect distinct subsets of activators to the transcriptional machinery resulting in different types of activated transcription (Chiang and Roeder, 1995). In other words, different activators, such as acidic, glutamine-rich, proline-rich, or isoleucine-rich activators, may interact with distinct TAFs in a single TFIID complex to modulate transcription in a specific way. For example, some types of activators in their contact with the PIC may influence the

initiation rate more than processivity, and other activators may contact other components of the PIC that produce the opposite effect. Experiments that argue against the idea that the degree of processivity is regulated by specific TAFs was obtained by studying the relationship between the number of activation domains (either within the same activator, or resulting from multiple activator binding sites in the promoter), and synergism of activation and processivity (Blair et al., 1996). The degree of processivity (type of transcription) was strictly dependent on the overall activity of the activator; low numbers of DNA binding sites, or activation domains resulted in relatively weak activation that showed little processivity.

Activators show very specific patterns of affinity with regard to TAFs and other components of the PIC. In *Drosophila*, glutamine-rich activators require contact with dTAF110 (Hoey et al., 1993), and acidic activators interact with dTAF40 (Goodrich et al., 1993; Thut et al., 1995). In humans, Sp1 (glutamine-rich activator) and VP16 (a typical acidic activator) activators require hTAF250 for transcriptional activation (Wang and Tjian, 1994). The estrogen receptor, but not VP16, interacts with hTAF30 (no-known *Drosophila* counterpart) (Jacq et al., 1994). Acidic activators also interact with hTAF31/32 which is the homolog of dTAF40 (Klemm et al., 1995). Multiple activators including Sp1, YY1, USF, CTF (proline-rich), E1A (acidic) and Tat can interact with hTAF55 (no-known homologue in

Drosophila) (Chiang and Roeder, 1995). Since hHSF1 contain both an acidic- and proline-rich activation domains, likely candidates for interactions include hTAF32 and hTAF55 based on the studies cited above.

Although TAFs have been shown to be essential in numerous examples of activated transcription, and serve as direct targets of many transcription activators in higher eukaryotes, a contrary view of the importance of TAFs in transcriptional activation has emerged recently in studies conducted in yeast (Moqtaderi et al., 1996; Walker et al., 1996). The complete inactivation of six yTAFs (TAF145, 90, 68, 60, 47 and 30) and the conditional shut-down of four yTAFs (TAF145, 90, 60 and 19) (Moqtaderi et al., 1996; Walker et al., 1996) had no effect on the transcriptional activation of several genes including Hsp70. Since yeast TAFs (yTAFs) are not the obligatory targets of transcriptional activators, other components in PIC, for example yeast TBP (yTBP), or yeast TFIIB (yTFIIB), may be the only universal targets of transcriptional activators. Because of the TAFs are highly conserved from yeast to humans, they must serve some essential function, perhaps in the cell cycle. One interpretation of the yeast finding is that perhaps TBP and TFIIB are major targets for activators in all eukaryotes, but in animals, the TAFs also serve in this capacity in addition to their other more essential roles.

Involvement of TFIIB in Transcriptional Activation

TFIIB has been cloned from archaebacteria, yeast, humans, *Xenopus*, and plants with aa identities ranging from 32-99% (Baldwin and Gurley, 1996; Pinto et al., 1992; Wampler et al., 1992). The core domain of human TFIIB (112-316) is highly conserved and is located in the C-terminal half of TFIIB. The core resembles cyclin A and interacts with the C-terminal stirrup of TBP through protein-protein interactions (Bagby et al., 1995). In addition, X-ray crystallography has demonstrated contacts between the core and the phosphoribose backbone of the TATA-box through protein-DNA interactions (Bagby et al., 1995; Nikolov et al., 1995). Alanine scanning analysis indicated that Core TFIIB also interacts with DNA upstream and downstream of TATA-box (Tang et al., 1996). The N-terminal domain of core TFIIB forms the downstream surface of the ternary complex where it may determine the transcription start site (Nikolov et al., 1995). The remaining surface of TFIIB, especially the N-terminus of TFIIB, as well as TBP, provides a stable platform for PIC assembly and interactions with TAFs, GTFs, activators and coactivators. The N-terminus of TFIIB (1-106), dispensable for TBP binding, is essential for recruitment of pol II to the DNA template and the interaction with TFIIF (Ha et al., 1993). Although the zinc finger is not important for interactions of TFIIB with other GTFs, it may provide a target surface for binding activator proteins For example,

the N-terminus of TFIIB, including a putative zinc finger and an adjacent charged region is required for ftzQ-mediated transcription activation (Colgan et al., 1995).

Because of TFIIB's central role in PIC assembly and transcriptional regulation, it serves as a point of communication among activators, TAFs, coactivators, and GTFs. The binding of TFIIB to the TFIID-TFIIA complex on promoters results in further stabilizing the PIC and provides a surface for the attachment of the pol II/TFIIF complex (Buratowski and Zhou, 1993; Choy and Green, 1993; Ha et al., 1993). Acidic activators, proline-rich and glutamine-rich activators can stimulate transcription by targeting TFIIB to the PIC *in vitro* and *in vivo* (Colgan et al., 1995; Colgan et al., 1993; Defalco and Childs, 1996; Hori et al., 1995; Kim and Roeder, 1994; Lin and Green, 1991; Roberts et al., 1993; Xiao et al., 1994). TFIIB mutants defective in activated, but not basal transcription, have been identified (Roberts et al., 1993). Affinities of TFIIB for activators show a strong correlation with transcription activating potentials (Wu et al., 1996) which suggest that TFIIB is a bona fide target for recruitment by transcription activators.

Activator interactions cause a dynamic conformational change of TFIIB. Structural and functional studies have shown that TFIIB has two domains: the C-terminal core domain (192 aa) containing two imperfect direct repeats capable of interacting with VP16, and an N-terminal domain (124 aa) containing a putative Zn-finger and possessing the ability to

interact with the C-terminal domain intramolecularly (Roberts and Green, 1994). In native TFIIB, the N- and C-terminal domains are engaged in an intramolecular interaction which can be disrupted by VP16. It has been postulated that acidic activators like VP16 function in a capacity beyond simple recruitment (Roberts and Green, 1994). Their disruption of the folded state of TFIIB in binding to the core may initiate a conformational change that exposes binding sites for other GTFs to facilitate the ordered assembly of the PIC.

Positive Cofactors in Transcriptional Activation

Besides the requirement for GTFs, transcriptional activation, in some cases, needs positive coactivators (PCs) as well. PCs contribute significantly to transcriptional activation in reconstituted mammalian transcription systems. Several members of a family of mammalian cofactors have been identified such as PC1, PC2, PC3/DR2/Topo I and PC4/p15. Of these, only PC3/DR2/Topo I and PC4 have been cloned (Ge and Roeder, 1994; Kretzschmar et al., 1993; Merino et al., 1993). A few positive cofactors have also been found in yeast (Berger et al., 1992; Swaffield et al., 1995). Among these, some appear to be SRB (suppressers of the CTD deletion of RNA polymerase B; B = II) proteins tightly associated with the CTD (C-terminal domain) of the largest subunit of pol II (Koleske and Young, 1994). The CTD contains up to 52 repeats of the heptapeptide YSPTSPS in human (Young, 1991).

PC2, a 500 kDa protein complex isolated from a HeLa cell cofactor fraction, is specifically required for activation by the artificial acidic activator GAL4-HA (Kretzschmar et al., 1994b).

In the absence of activators, PC3/DR2/Topo I represses basal transcription *in vitro*, a condition that can be overcome by addition of TFIIA (Kretzschmar et al., 1993; Merino et al., 1993). This observation is led to the hypothesis that a function of TFIIA is to strip away repressors present in the TFIID (Cortes et al., 1992). However, in the presence of activators including acidic-, proline-, and glutamine-rich activators, it increases activator-mediated transcription to levels higher than these expected to result simply from the removal of repressors (Kretzschmar et al., 1993; Merino et al., 1993). Both the activation and inhibitory effects of PC3 are specific for TATA-element-containing promoters (Merino et al., 1993). Since PC3 can be copurified with hTFIID fraction, and its direct interaction with hTBP has been demonstrated by far-western analysis, PC3 may function as a positive cofactor through direct interaction with TBP (Merino et al., 1993). It also contains DNA topoisomerase I activity which is dispensable for transcriptional repression, activation and elongation (Merino et al., 1993).

PC4 substantially increases the amplitude of activator-dependent activation with representatives of all types of transcription activators including acidic (GAL4), proline-

rich (CTF), glutamine-rich (Sp1) activators indicating its general function in transcriptional activation (Ge and Roeder, 1994). The N-terminus of PC4 contains a SEAC motif, a serine-rich and an acidic aa-rich region which is essential for its coactivator function (Kaiser et al., 1995). It also has phosphorylation sites for casein kinase II (CKII), which is a protein that represses cofactor activity (Kaiser et al., 1995; Kretzschmar et al., 1994a). The phosphorylation of PC4 by CKII can abolish interactions of PC4 with the TBP-TFIIA-promoter complex, activators, and disrupt its cofactor function (Ge et al., 1994; Kretzschmar et al., 1994a). PC4 can interact with VP16 and TFIIA simultaneously (Ge et al., 1994). This bridging function may serve to recruit TFIIA for stabilization of TFIID binding. The C-terminal domain has single-strand and double-strand DNA binding abilities whose precise roles are unclear. However, its double strand DNA binding ability correlates with its capacity to facilitate activation (Kaiser et al., 1995). PC4 contains a lysine-rich region that is responsible for nonspecific DNA binding.

In summary, regardless of the exact mechanism of activator-mediated transcription, physical contact between activators and components of the PIC is a critical early step in this process. Since TBP, TFIIB, TAF32, TAF55 and PC4 are very important targets for various transcriptional activators, it seems worthwhile to characterize potential interactions between HSFs and these four components of the

PIC *in vitro*, as well as test the functional significance of these interactions *in vivo*.

MATERIALS AND METHODS

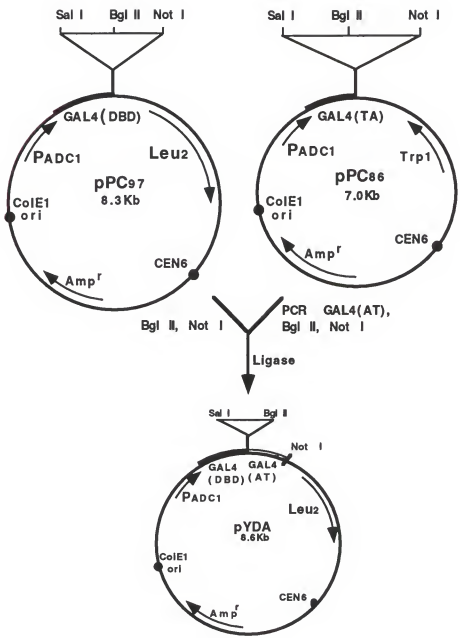
Yeast Strains and Growth

Yeast strain PCY2 (*MATα gal4 gal80 URA3::GAL-lacZ lys2-801 his-200 trp1-63 leu2 ade2-101*) was used to assess β-galactosidase (β-gal) activity of all constructs containing the GAL4-DBD fused to the N-terminus of the mutant HSFs (Chevray and Nathans, 1992). Strain YJB341(*a/trp1-289[TRP1 HSE-lacZ] ura3-52 leu2-3, 112 his31/his3HindIII met2/MET2 his4-519/HIS4 ade1-100/ADE1 hsf1gal^{-}/gal^{+}* [YEpHSF^{RS}URA]) contains the scHSF on a YEplac vector and was used to substitute the heterologous HSFs from soybean and humans (Bonner et al., 1992). This strain also contains an integrated *HSE-lacZ* gene for monitoring HSF activity. The plasmid pBS16$_{M232V}$ was grown in strain YJB341 (designated 341/BS16$_{M232V}$) and contained a VP16 fusion with the N-terminus of scHSF$_{M232V}$ in a pDBD vector (Bonner, 1991). The point mutation of methionine at residue 232 makes the HSF constitutive (Bonner et al., 1992).

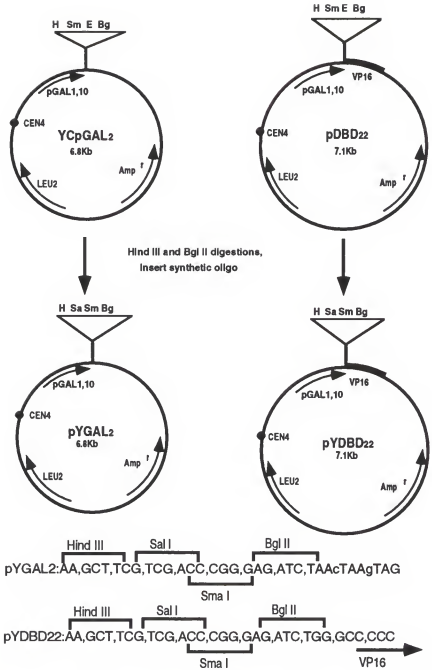
Yeast Vectors and their Derivatives

Plasmid pPC97, a GAL4-DBD fusion vector (Vector 1), is an improved version of the vector pPC62 (personal communication; P. M. Chevray), and differs only in the

polylinker sequence which is identical to that in pPC86 (Chevray and Nathans, 1992). Plasmid pPC86 was used as template to PCR-amplify the GAL4 activation domain (GAL4-AD, aa 768-881) via specific primers. The upstream primer contained a *Bgl* II site and the downstream primer a *Not* I site. The PCR product corresponding to the GAL4-AD was subcloned into pPC97 between *Bgl* II and *Not* I sites to generate vector plasmid pYDA (Vector 1) containing both the GAL4-DBD (N-terminal) and the GAL4-AD (C-terminal) with a site for insertion of modified HSF sequences in between. The open reading frame was maintained in the GAL4-DBD-AD fusion protein of pYDA. Plasmids derived from YCpGAL2 (without VP16) and pDBD22 (VP16 C-terminal fusion vector) were used to shuttle heterologous HSFs into yeast for HSF substitution experiments (Bonner, 1991). The polylinkers of these two plasmids were modified to facilitate the cloning of HSFs by the substitution of *Sma* I and *Eco* RI sites with *Sal* I and *Sma* I sites by insertion of a synthetic oligonucleotide to create plasmids pYGAL2 (no VP16) and pYDBD22 (VP16 fusion) (Vector 2). The sequences of the new polylinkers are: 5'-AA,GCT,TCG,TCG,ACC,CGG,GAG,ATC,TAA,C,TAA,G,TAG-3' for pYGAL2; and 5'-AA,GCT,TCG,TCG,ACC,CGG,GAG,ATC,TGG,GCC,CCC-3' for pYDBD22. Yeast strain PCY2, and vector plasmids pPC97 and pPC86 were kindly provided by Dr. P. M. Chevray (The Johns Hopkins Univ.). Yeast strain YJB341, vector YCpGAL2 and pDBD22 were kindly provided by Dr. J. J. Bonner (Indiana Univ.).



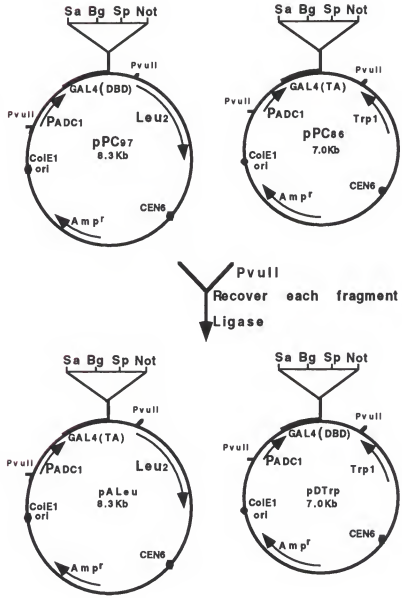
Vector 1. pYDA is a yeast expression vector used in the studies of basal repression of HSFs. The vector was derived from pPC86 and pPC97 (Chevray and Nathans, 1992).



Vector 2. pYGAL2 and pYDBD22 are yeast expression vectors with the GAL1, 10 promoter used for the substitution of scHSF with HSFs from human and soybean. Both vectors were derived from YCpGAL2 and pDBD22 (Bonner, 1991).

Construction of HSF Mutants in Yeast Vectors

The cDNA clones containing hHSF1 and hHSF2 were kindly provided by Dr. Carol Wu and Dr. Robert Kingston. Nested deletions of hHSF1, hHSF2, GmHSF5, and GmHSF34 were generated by PCR using Vent DNA polymerase (New England Biolabs) according to the manufacturer's instructions. A series of specific downstream and upstream primers with added *Sal* I and *Bgl* II sites, respectively, were synthesized and used in PCR-mediated amplification reactions. The PCR products were digested with *Sal* I and *Bgl* II and subcloned directionally into yeast expression vectors pPC97, pPC86, pYDA, pYGAL2 and pYDBD22. Modified HSFs cloned into vectors pPC97 (GAL4-DBD fusions) and pYDA (GAL4-AD fusions) were transformed (Ausubel et al., 1991b) into yeast strain PCY2, and those in vectors pYGAL2 or pYDBD22 were transformed into strain YJB341. Transformants from strain YJB341 were further selected on 5-fluoroorotic acid (5-FOA) plates which ensured loss of the endogenous scHSF contained on the URA3 vector plasmid. Yeast strain YJB341 was grown in yeast medium supplemented with 2% galactose, and PCY2 was grown in medium containing 2% glucose. For the two-hybrid system assay, two constructs in pPC86 and pPC97 were co-transformed into PCY2 and selected on Leu and Trp double drop-out (Leu and Trp minus) medium.



Vector 3. pALeu and pDTrp are yeast expression vectors used in yeast two-hybrid system for cDNA screening. Both vectors were derived from vector pPC86 and pPC97 (Chevray and Nathans, 1992).

Yeast Two-Hybrid System Screening

All manipulations of the yeast two-hybrid system screening were done according to the protocol supplied by Clontech. The HeLa S3 matchmaker cDNA library (Clontech) in yeast expression vector pGAD-GH (GAL4-AD fusion vector with Leu2 selection marker) was amplified and the DNA purified by CsCl ultracentrifugation. A C-terminal deletion construct (CTA1-deletion, aa 1-422) of hHSF1 was cloned into the *Sal* I and *Bgl* II sites of yeast expression vector pDTrp (Vector 3) (GAL4-DBD fusion vector with Trp1 selection marker) derived from pPC97 and pPC86. This plasmid (the bait vector) and the cDNA library were co-transformed into yeast strain HF7c and selected on His-drop-out (His minus) plates supplemented with 20 mM 3-amino-triazole (3-AT). Positive clones were re-selected by filter assay of β-gal according to the protocol supplied by Clontech. Clones passing through the double selections were grown on Leu-drop-out medium to maintain the vector plasmid containing the cDNA insert and lose the bait plasmid. DNA of the cDNA clones were isolated and co-transformed with the bait plasmid to eliminate false positives. Only the clones that required the bait plasmid for β-gal expression were selected for further characterization. Clones passing these tests were subcloned into pUC19 and DNA sequenced (ICBR sequencing core of UF).

Site-Directed Mutagenesis

For site-directed mutagenesis and internal deletion of GmHSF34, the full length GmHSF34 was subcloned into the *Sal* I and *Bam* H1 sites of phage M13mp18. Mutagenesis was conducted using the Muta-gene M13 *in vitro* mutagenesis kit (Bio-Rad) except that Klenow enzyme was used to replace the T4 DNA polymerase in the synthesis of the second strand. The mutants were selected by restriction enzyme digestion at a site supplied by the primer, and the sequence of the mutants was confirmed by DNA sequencing. The modified GmHSF34 coding regions were subcloned into pYDA and transformed into yeast PCY2. The β-gal activities of the mutant HSFs were determined at both basal (25°C) and heat shock (37°C) conditions.

PCR-Mediated Random Mutagenesis

Low fidelity PCR random mutagenesis (Kassenbrock et al., 1993) was used to generate random mutants of the C-terminal region (AD2, aa residues 464 to 529) of hHSF1. An upstream primer with a *Sal* I site and a downstream primer with a *Bgl* II site were used to PCR-amplify aa residues 464 to 529 of AD2 for random mutagenesis. The PCR products were digested with *Sal* I and *Bgl* II, and subcloned into pPC97. The mixed population of mutants were transformed into yeast strain PCY2 to form a mutant library. Mutations that affected transcriptional activity were screened either by a filter

assay, or a liquid assay of β-gal according to the yeast two-hybrid system protocol (Clontech). All mutant yeast colonies were pooled and their DNAs were isolated and transformed into *E.coli* strain DH5α. Each mutant was reisolated and reintroduced into yeast strain PCY2. Their reduced level of transcriptional activity was reconfirmed by the β-gal liquid assay. All the confirmed mutants were sequenced by using a specific primer located in the GAL4-DBD just upstream of the polylinker.

Heat Shock Treatment and β-gal Assay in Yeast System

Temperatures of 25°C (non-heat shock), 37°C and 40°C were used to stress the yeast cultures for periods of time from zero to 20 hr after the OD600 of the cultures reached approximately 0.5. After temperature treatments, β-gal assays were performed. These experiments were repeated three times. In order to test the survival of exogenous-HSF-substituted strains under hs and cold stress conditions, the transformants selected from 5-FOA plates were spread on Leu drop-out and galactose-containing yeast medium and incubated at 15°C, 30°C and 37°C.

Western blot Analysis of GmHSF in Yeast

Cells from 1.25 ml cultures (OD_{600} = 1.0) were pelleted and lysed by addition of 160 µl of fresh 1.85 M NaOH/7.4% 2-mercaptoethanol and kept on ice for 10 min. Proteins were precipitated by addition of 160 µl of 50% (wt/vol)

trichloroacetic acid and kept on ice for 10 min. The proteins were collected by spinning 2 min in an Eppendorf centrifuge and washed with 1 ml of ice-cold acetone. The air-dry proteins were re-extracted at 94°C for 5 min with 150 µl of 2x SDS loading buffer (Ausubel et al., 1991a). The soluble proteins (ca. 40 µg/lane) were separated by 8% SDS-PAGE, and the resolved proteins were transferred to a nitrocellulose membrane by electrophoresis. The ProtoBlot Western blot AP System from Promega was used to detect the expressed GmHSF34 in yeast. A 250-fold dilution of the GmHSF34 polyclonal antibody serum was used to probe the western blot.

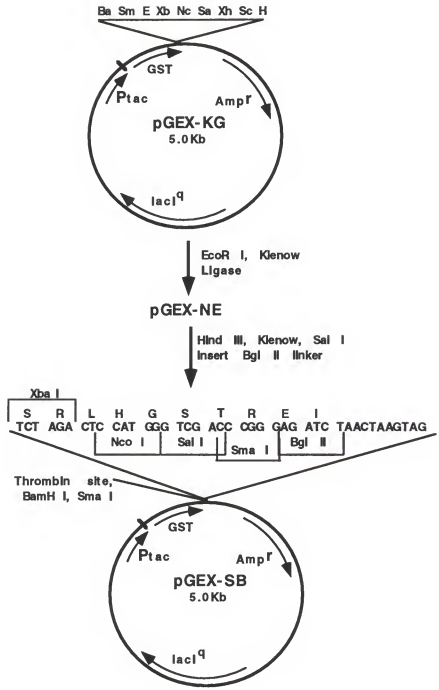
Production of Antibodies against GmHSF34

The full length clone of the soybean HSF, GmHSF34, was subcloned into the pET15b vector (Novagen, Inc.). The clone was transformed into *E. coli* BL21-DE3, induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and grown at 37°C for 3 hr. The GmHSF34 protein was extracted from inclusion bodies with 1.5% N-lauroyl sarcosine plus 1 mM ethylenediaminetetraacitic acid (EDTA) and purified by Ni column chromatography (Frankel et al., 1991; Hochuli et al., 1987). Protocols for preparation of inclusion bodies and Ni column purification of histidine tagged proteins were provided by Novagen. Recombinant proteins were further purified by 8% SDS-PAGE with the band envisioned by precipitation in the gel with cold 0.25 M KCl. Recovered

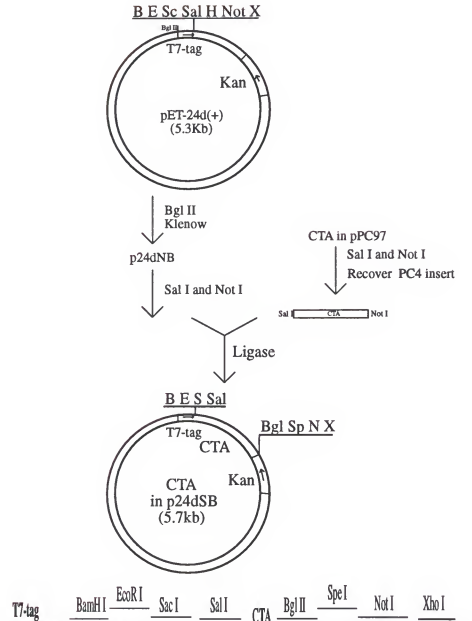
bands containing 300 µg of GmHSF34 protein were lyophilized for 2 days and pulverized for injection into mice. For antibody production, two mice received triple injections at 50 µg of protein per injection.

GST-Fusions and the Pull-down Assay

Glutathione-S-transferase (GST) fusion vector pGEX-SB was derived from pGEX-KG (Guan and Dixon, 1991) by filling the *EcoR* I site and inserting a *Sal* I and *Bgl* II linker between *Sal* I and *Hind* III sites. All fusions including hHSF1 and 2, hTBP, hTFIIB, as well as their deletions, were constructed between *Sal* I and *Bgl* II sites in expression vector pGEX-SB (Vector 4) and expressed in *E.coli* strain BL-21. Expression of the fusion proteins were induced by 0.1 mM IPTG at room temperature for 3 hr and purified by Glutathione Sepharose 4-B beads according to the Pharmacia protocol (commercial supplier). The purified fusion proteins were stored in Buffer A(100) without BSA. Buffer A(100) contained 1.25 ml of 1M HEPES, pH 7.5, 0.25 ml of 1 M $MgCl_2$, 12.5 µl of 0.4 M EDTA, 6 ml of glycerol, 0.37 g of KCl, 2 µl of 2-mercaptoethanol, 50 µl of NP-40 and 50 µl of 4% bovine serum albumin (BSA) in 50 ml. The amount of protein on the bead was estimated by SDS-PAGE and Coomassie Brilliant Blue staining. GST-VP16(413-490) and GST-VP16(Δ456-FP442) were kindly provided by Dr. S. G. E. Roberts (Roberts and Green, 1994) as positive and negative controls.



Vector 4. pGEX-SB is a GST fusion protein vector for expression in *E. coli*. It was derived from pGEX-KG (Guan and Dixon, 1991).



Vector 5. p24d-SB is a *E. coli* expression vector with the T7-epitope. It was derived from pET-24d(+) (Novagen).

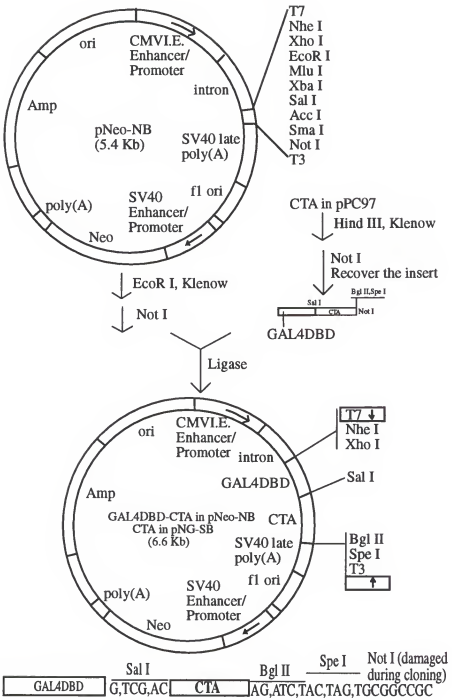
Transcription factors and derived protein fragments were translationally fused with the T7-epitope to allow detection by western blot. T7-tagged hTFIIB in pET5a (Novagen) was provided by Dr. S. G. E. Roberts. hTBP from Dr. R. G. Roeder was tagged by cloning into *Sal* I and *Bgl* II sites of vector p24d-SB (Vector 5) which was derived from pET24d (Novagen) by blocking *Bgl* II and inserting a stuffer DNA fragment with *Sal* I site upstream and *Bgl* II downstream. Three cDNA clones, PC4, hTAF32 and hTAF55, in addition of the CTA1-Plus fragment of hHSF1 were cloned from the HeLa S3 matchmaker cDNA library (Clontech) by PCR via gene-specific primers and tagged with the T7-epitope in vector p24d-SB. All of these T7-tagged clones were expressed in *E.coli* strain BL-21(DE3) by 0.1 mM IPTG induction for 3 hr at room temperature. The bacterial pellets from a 50 ml culture (OD600=1-2) were suspended in 3 ml Buffer A(100) and sonicated on ice. The bacterial protein lysates were collected after a 5 min centrifugation (microcentrifuge) at 4°C and stored at -20°C.

The binding assays were performed in 100 µl of buffer A(100, 150 or 300), containing about 5 µg of GST-fusion proteins on beads and 5 µl of T7-tagged protein lysate. The binding incubation was 2 hr at 4°C with gentle rocking. The beads were extensively washed with washing buffer which is a derivative of Buffer A(150) with 10-fold less BSA. The beads were boiled in 40 µl of 1XSDS loading buffer, and the proteins were resolved by 10% SDS-PAGE. Epitope-tagged proteins were visualized by western blot using a 1:10,000

dilution of anti-T7-tag monoclonal antibody (Novagen) coupled with ECL detection system (Amersham).

HeLa Cell Transfection and Heat Shock Treatment

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum at 37°C and transfected by the calcium phosphate precipitation method (Ausubel et al., 1991c). 0.5 µg of Gal4-DBD-hHSF1 expression constructs in vector pNG-SB (Vector 6) (derived from pCI-neo vector from Promega Corporation), 0.5 µg of human growth hormone reporter (pXGH5 from Nichols Institute Diagnostics) for transfection efficiency, and 2 µg of pG5Luc luciferase reporter DNA (Patel et al., 1995) were used in each transfection. Cells were harvested after 48 hr, and transfection efficiency was estimated with the hGH-TGES 100T Kit (Nichols Institute Diagnostics). Luciferase activity of the reporter was measured using a commercially supplied kit (Luciferase assay system with reporter lysis buffer, Promega Corporation). Since hs inhibits enzymatic activity of luciferase, allowing for the recovery of HeLa cells at 37°C after hs was required in order to measure activity. Forty four hr after transfection, HeLa cells were incubated for 2 hr at either 42°C (hs) or 37°C (control), and allowed to recover for 2 hr at 37°C before harvesting.



Vector 6. pNG-SB and pNeo-NB are mammalian expression vectors. pNG-SB contains GAL4-DBD for the production of GAL4-DBD fusion proteins. pNeo-NB lacks the GAL4-DBD. Both vectors were derived from pCI-neo vector (Promega).

HeLa Cell Nuclear Extract and Far-Western

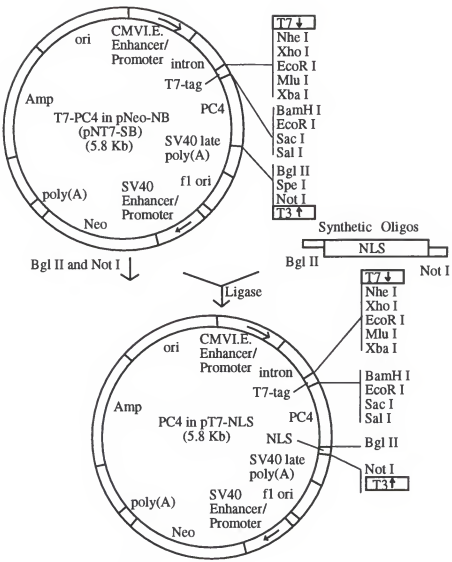
HeLa cell nuclear extracts were prepared according to the method of Dignam (Dignam et al., 1983) using 1X 10^9 cells. 500 µl of nuclear extract and 100 µl of GST-CTA1 immobilized on Sepharose-4B beads was used for the interaction of CTA1 with nuclear proteins in buffer A(100). Interaction was allowed to occur for 2 hr at 4°C with gentle rocking. The beads were then washed twice with 2 ml of buffer A(100) each time and bound proteins eluted with buffer A(500) (buffer A with 500 mM KCl). The eluted proteins were precipitated by cold 12% TCA and separated by SDS-PAGE before transfer to Immobilon PVDF membrane (Millipore). The blotted membranes were incubated with T7-tagged CTA1 in buffer A(100) overnight at 4°C. Washed membranes were then crosslinked for 30min at 4°C in buffer A(100) containing 0.5% glutaraldehyde. The membranes were probed by monoclonal antibody against the T7-tag, then visualized using ECL (enhanced chemiluminescense).

The Squelch-Rescue Assay for Analysis of *in vivo* Interactions

An assay based on the squelching of activated transcription was utilized. As a test for *in vivo* protein-protein interactions between the activation domain of hHSF1 (combined AD1 and AD2, designated as CTA1-Plus) and various proteins that comprise the PIC. In this assay, the activity

of a GAL4-DBD fusion with the CTA1-Plus fragment (activation domain AD1 and AD2) was squelched in transient assays by co-transfection with vectors incorporating dysfunctional constructs of the potentially interacting proteins. If the test factor bound the GAL4-DBD-CTA1-Plus protein, a dysfunctional complex would be formed resulting in a decrease, or squelching, of luciferase activity of the reporter which was driven by a promoter containing the four GAL4 DNA binding sites. This squelching should be alleviated, or the luciferase activity of the reporter increased, if a second mutation was introduced into the dysfunctional factor that eliminated its interaction with the CTA1-Plus construct. This strategy of squelch and squelch-rescue was previously employed in the characterization of hTFIIB interactions with a glutamine-rich activator (Colgan et al., 1995) and in a demonstration of interaction between p53 and TFIIB and TFIID (Liu and Berk, 1995).

A series of transcriptionally dysfunctional mutants of transcription factors including hTBP, hTFIIB, hTAF32 and hPC4 were cloned into the *Sal* I and *Bgl* II sites of the modified mammalian expression vector pT7-NLS (Vector 7) (derived from pCI-neo vector). The pT7-NLS vector has two unique features for the squelch-rescue assay: a T7-tag for the detection of protein expression, and a nuclear localization signal (NLS) for transporting expressed proteins into the nucleus. This NLS contains 13 aa residues from human DNA ligase I (Montecucco et al., 1995). HeLa cells were co-



T7-tag BamH I EcoR I Sac I Sal I PC4 Bgl II NLS Not I

GGT,CGG,ATC,CGA,ATT,CGA,GCT,CCG,TCG,AC AG,ATC,T GCGGCCGC

P K R R T A R K Q L P K R

NLS: 5'G,ATC,TTT,CCG,AAG,CGT,CGC,ACA,GCT,CGG,AAG,CAG,CTC,CCG,AAA,CGG,C3'
3'AAA,GGC,TTC,GCA,GCG,TGT,CGA,GCC,TTC,GTC,GAG,GGC,TTT,GCC,GCC,GG5'

Vector 7. pT7-NLS is a mammlian expression vector with T7-tag and nuclear localization signal. It encodes both a T7-tag and NLS (Montecucco, et al 1995) used in squelch-rescue assays. It was derived from the pCI-neo vector indirectly via pNT7-SB.

transfected with four plasmids: 3 μg of the transcriptionally dysfunctional mutants in the pT7-NLS vector, 2 μg of reporter plasmid pG5luc containing GAL4 binding sites upstream of luciferase gene, 0.5 μg of the effector construct containing GAL4-DBD-CTA1-Plus fusion in the pNG-SB vector, and 0.5 μg of pXGH5 for monitoring transfection efficiency. The final amount of DNA for each transfection was equalized by adding empty vector pNeo-NB DNA where appropriate. All transfections were conducted in triplicate, and activities of the luciferase reporter gene were normalized for transfection efficiency by dividing units of luciferase activity by units of human growth hormone produced.

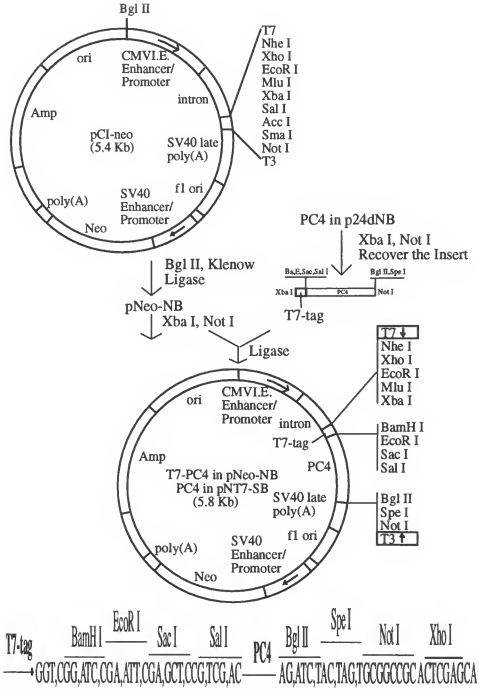
Co-immunoprecipitation

A second method used to detect *in vivo* associations between the activation domains of hHSF1 and GTFs was based on their co-immunoprecipitation. CTA1, mutated CTA1 and CTA1-Plus constructs of hHSF1 in the GAL4-DBD vector pNG-SB were each co-transfected with T7-tagged hTBP (also in vector pNT-SB) into HeLa cells. CTA1 and T7-hTBP were transfected individually into HeLa cells as negative controls. Forty eight hr after transfection, cells were harvested and lysed by three rounds of freezing-and-thawing in buffer A(500) plus a cocktail of proteinase inhibitors (0.1 mM PMSF, 2 μg/ml leupeptin, 3 μg/ml pepstatin A, 40 μg/ml antipain). The lysates were cleared by centrifugation for 5 min at 4°C and collected. For co-immunoprecipitation, the lysates were

diluted with buffer A(0) to the same final salt concentrations as in buffer A(150). GAL4-DBD fusions were co-immunoprecipitated from cellular lysate with anti-GAL4-DBD monoclonal antibody (mAb) which was covalently crosslinked to protein A Sepharose beads by using DMP (dimethylpimelimidate) (Harlow and Lane, 1988). The beads were washed twice with buffer A(150) and boiled for 5 min in SDS loading buffer. Released proteins were resolved by 10% SDS-PAGE and blotted to Immobilon PVDF membrane (Millipore). The membrane was then probed with mAb (monoclonal antibody) against the T7-epitope to detect the T7-tagged hTBP.

Pull-down Assay of hTBP Deletions Transiently Expressed in HeLa Cells

hTBP and its deletions were cloned into *Sal* I and *Bgl* II sites of vectors pNT7-SB (Vector 8) (without NLS for wild type TBP) or pT7-NLS (with NLS for deletions of TBP) and transiently expressed in HeLa cells. Cells were harvested and lysed in buffer A(500) plus proteinase inhibitors by three rounds of quick freezing and thawing. The lysates were collected after a 10 min centrifugation (microcentrifuge) at 4°C. The lysate was incubated with rocking for 2 hr at 4°C with 5 μg of GST-CTA1 or GST-CTA1-Plus fusion protein immobilized on Sepharose-4-B beads in buffer A(150). The beads were washed three times with buffer A(150) and boiled for 5 min in SDS loading buffer. Released proteins were analyzed by western blotting as described above.



Vector 8. pNT7-SB is a mammlian expression vector with the T7-tag. It was derived from the pCI-neo vector (Promega).

hTFIID Complex Pull-down by GST-fusions of hHSF1

For detection of interactions of CTA1 and CTA1-Plus with hTFIID complex, 180 µl of buffer A(100) plus 0.1% NP-40, 50 µl of the HeLa nuclear extract and 20 µl of immobilized GST-CTA1, or GST-CTA1-Plus, were incubated for 2 hr at 4°C with gentle rocking. The final salt concentration was adjusted to 100 mM, 150 mM or 250 mM by adding 3 M KCl. The beads were washed twice with buffer A(150) and then eluted by buffer A(500) and visualized by western blotting. Endogenous hTBP and TAF250 in the TFIID complex were detected by anti-hTBP mAb from Promega and anti-TAF250 mAb provided by Dr. R. G. Roeder. For detection of interactions between NR and the TFIID complex, 500 µl of buffer A(100) and 500 µl HeLa nuclear extract were mixed and passed through GST-NR affinity column (150 µl of GST-NR) by gravity. Bound proteins were washed with 1.5 ml of buffer A(100), eluted with buffer A(500) and visualized by western blot using anti-hTBP antibody.

RESULTS

The Function and Structure Studies of HSFs from Human and Soybean

Mapping the Transcriptional Activation Domains of hHSF1 and hHSF2 in Yeast and HeLa Cells

In order to identify domains of hHSF1 involved in transcriptional activation and maintenance of basal repression, a series of N- and C-terminal truncations were initially evaluated in yeast cells. Activity of hHSF mutants was monitored using β-gal activity generated by a GAL1-*lacZ* reporter integrated into the chromosome of yeast strain PCY2. All hHSF constructs were fused with the GAL4-DBD at the N-terminus (plasmid pPC97, Vector 1). Since nuclear localization and DNA binding functions were provided by the GAL4-DBD, this system tolerated mutations which eliminated the corresponding regions of the hHSF. Mutations could also be placed in the OD since HSF trimerization was presumably not needed for binding to the GAL4 DNA binding site. Expression of the full length hHSF1 protein fused to the GAL4-DBD showed activity in yeast under hs conditions and no detectable activity under normal growth conditions indicating that basal repression and heat inducible activation of hHSF1 were able to occur in this heterologous system (Fig. 1,

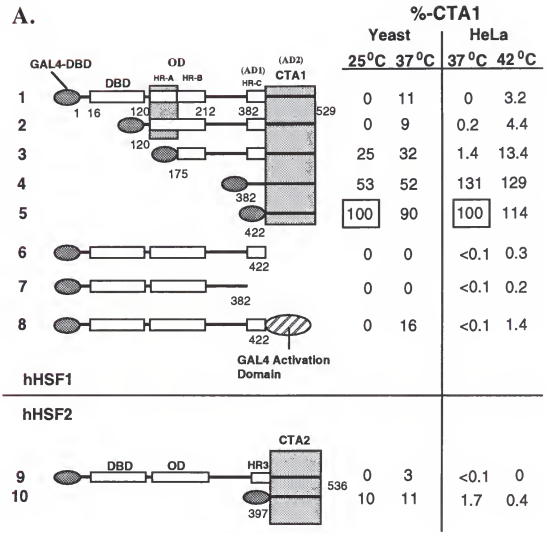


Fig. 1. Mapping of human HSF1 and HSF2 regions involved in transcriptional activation and basal repression in yeast and HeLa cells. The transcriptional activity of construct 5 under non-heat shock conditions was designated as 100% in both yeast and HeLa cells, and the activities of other constructs were expressed as relative activity of construct 5. CTA1 = C-terminal transcriptional activation domain of hHSF1; CTA2 = C-terminal transcriptional activation domain of hHSF2; DBD = DNA binding domain; HR =heptad repeat; OD = oligomerization domain.

construct 1). Removal of 119 aa residues (aa) from the N-terminus demonstrated that the hHSF1 DBD was not involved in basal repression or transcriptional activation (construct 2). Further deletion of aa 120 to 174, which removed HR-A of the OD, resulted in both an increase in overall activity and a loss of basal repression (construct 3). Activity under both control and HS conditions nearly doubled when the remaining portion of the OD (HR-B) and the region N-terminal to HR-C was deleted in construct 4 despite low levels of protein expression (Fig. 2). Loss of transcriptional activity was not seen until a portion of the C-terminus was deleted in construct 6. These results indicate that, when assayed in yeast cells, the transcriptional activation domain of hHSF1 is located in the 107 aa C-terminal to HR-C. The difference in β-gal activity between constructs 4 and 5 was most likely artifactual due to the extremely low amount of stable protein derived from construct 4 (Fig. 2). All other constructs showed similar levels of expression and correct protein size by western blot analysis (Fig. 2). The C-terminal activation domain identified here in yeast corresponds to the AD2 region characterized by Shi *et al.* (Shi et al., 1995) and Newton *et al.* (Newton et al., 1996) and does not include the adjacent region surrounding HR-C (AD1 domain) (Green et al., 1995; Newton et al., 1996; Zuo et al., 1995).

The same experiment was repeated in HeLa cells with similar results except in the case of construct 3. Comparison of the activity of construct 3 in yeast and HeLa

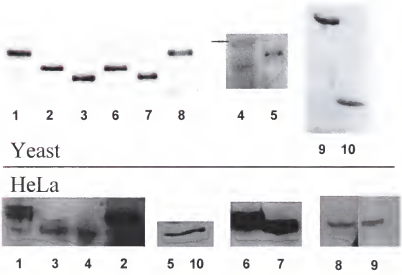


Fig. 2. Western blot of human HSF constructs expressed in yeast and HeLa cells probed with anti-GAL4-DBD antibodies. The bar indicates expected size of expressed protein for construct 4. Numbers correspond to constructs depicted in Fig. 1.

cells, under both hs and non-heat shock conditions, indicated that HR-A was required for the basal repression of hHSF1 in yeast, but dispensable in HeLa cells (Fig. 1). Similar levels of protein expression in HeLa cells were observed for each of the constructs except constructs 2, 6 and 7 (Fig. 2). There was no correlation between the efficiency of protein expression and activity of the reporter genes. For example, constructs 6 and 7 showed high level of protein expression, but only residual activity. The low activity of construct 6 was not surprising since the CTA1 domain had been deleted.

A possible mechanism for basal repression may include masking of the DBD under non-heat shock conditions. To test this hypothesis, gel retardation assays were performed using whole cell extracts from the strain containing construct 2 to determine if basal repression was due to the inability to bind DNA under control conditions. Analysis of extracts from construct 2 grown under non-heat shock conditions indicated that the GAL4-DBD was able to bind DNA despite the lack of transcriptional activity (Fig. 3). These results indicate that the GAL4-DBD was not masked during basal repression under non-heat shock conditions, and imply that the DBD of the wild-type protein may also be accessible under basal conditions. The slight reduction in DNA binding activity upon hs may reflect a partial loss of function of the GAL4-DBD. The presence of the GAL4-DBD-hHSF1 protein in the DNA complexes was confirmed by the lack of complex formation by the vectorless control strain PCY2, and by removal of

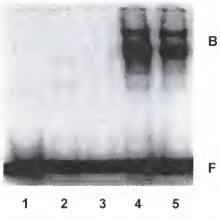


Fig. 3. Gel retardation assay of whole cell extract prepared from yeast containing construct 2. The GAL4-DNA binding site was used as a probe to test the ability of construct 2 to bind to DNA under control and heat shock conditions. Lane 1 is free probe. Strain PCY2 containing no vector plasmid was loaded in lanes 2 (non-heat shock) and 3 (heat shock). Whole cell extracts containing construct 2 was loaded in lanes 4 (non-heat shock) and 5 (heat shock). The presence of GAL4-DBD fusion proteins in the bound complexes was confirmed by supershift using monoclonal antibodies against the GAL4-DBD (not shown). F = free probe; B = bound.

complexes by immunoprecipitation with antiGAL4-DBD antibody (not shown).

The C-terminal location of the activation domain of hHSF2 (aa 397 to 536, designated as CTA2) is similar to the CTA1 of hHSF1 as demonstrated by constructs 9 and 10 (Fig. 1). Although activity of full length hHSF2 was only one fourth that of hHSF1 in yeast, basal repression and heat inducibility were still evident. As with hHSF1, removal of all portions of the HSF N-terminal to HR-C resulted in a large increase (4-fold) in constitutive activity. The CTA1 of hHSF1 (AD2) appeared to be approximately eight times stronger than CTA2 of hHSF2 when assayed in isolation in yeast cells. HSF2 constructs 9 and 10 showed little activity in HeLa cells compared to yeast. Full length HSF2 had no significant activity under either basal or hs conditions. In addition, activity of CTA2 (construct 10) was less than 2% of CTA1, whereas in yeast this domain exhibited between 10 and 11% of CTA1 activity.

Experiments were conducted to evaluate potential interactions between hHSF1 and hHSF2 activation domains since activation of the Hsp70 gene appears to be synergistically induced by hHSF1 and hHSF2 in cells (Sistonen et al., 1994). GAL4-DBD fusion proteins containing either CTA1 of hHSF1, or CTA2 of hHSF2 were co-expressed and activity monitored using the GAL4 DNA binding site reporter. As shown in Fig. 4, activity of CTA1 (construct 5), was approximately 65-fold greater than CTA2 (construct 10) when each was expressed

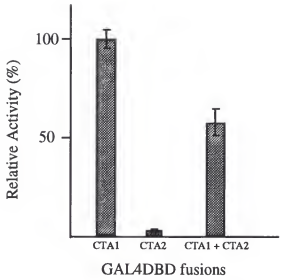


Fig. 4. Co-expression of GAL4-DBD fusion proteins containing the CTA1 or CTA2 of hHSF1. GAL4-DBD fusion proteins were expressed in HeLa cells using vector pNG-SB. DNA used in transfections: lane 1, CTA1 (0.5 µg, construct 12); lane 2, CTA2 (0.5 µg, construct 10); lane 3, CTA1 (0.5 µg) plus CTA2 (0.5 µg). Each transfection contained 0.5 µg of growth hormone vector (pXGH5) and 2 µg of reporter plasmid (pG5Luc). The amount of DNA per transfection was kept constant by adjusting transfections (lanes 1 and 2) with the addition of "empty" vector DNA(0.5 µg of pNeo-NB). Relative luciferase activity was normalized using growth hormone production. Error bars were derived from three replicate experiments.

alone. Co-expression of CTA1 and CTA2 resulted in activity levels that were intermediate between that of the two activation domains expressed separately indicating a lack of synergistic interaction. These results suggest that the synergistic response seen previously in hemin and heat induced K562 cells must involve events not directly related to the C-terminal activation domains of hHSF1 and 2.

Two interesting phenomena (Fig. 1) are evident in the comparison of activities between either the full length protein (construct 1), or the isolated CTA1 domain (construct 5) and a C-terminal deletion of CTA1 which leaves AD1/HR-C intact (construct 6). The first is the amazingly low activity level of the full length protein compared to the isolated CTA1 in both HeLa and in yeast. This result indicates that CTA1 is heavily repressed in the full length protein, even under hs conditions. A second point of interest is the total lack of activity of AD1/HR-C when CTA1 has been removed, but the remainder of the protein is present (construct 6).

Identification of Subdomains within the Transcriptional Activation Domain of hHSF1

Deletions within CTA1 were conducted to more precisely define the aa motifs involved in transcriptional activation (Fig. 5). The conservation of CTA1 subdomains is shown in Fig. 6. Three subdomains were identified that make substantial contributions to activity: subdomain I located between aa 422

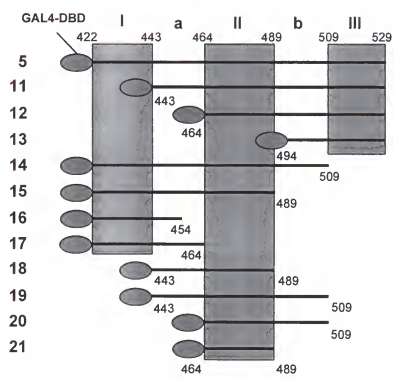


Fig. 5. Subdomain mapping of CTA1 in yeast. All constructs were derived from pPC97 and were expressed in yeast strain PCY2. A.) Major regions of activity are designated I, II, III with minor regions designated a and b. B.) Western blots of human HSF1 constructs expressed in yeast using anti-GAL4-DBD antibody as probe. Numbers correspond to constructs depicted in panel A. NHS = non-heat shock; HS = heat shock.

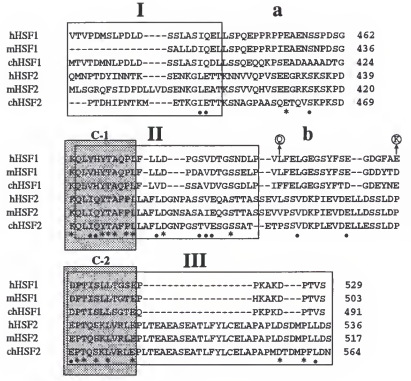


Fig. 6. Comparison of the CTA1 of hHSF1 with C-terminal regions of vertebrate HSFs. Amino acid sequences were compared using the CLUSTAL W(1.4) program. Identical residues are designated by an asterisk and those with conserved biochemical character are indicated by a closed circle. Major subdomains of activity determined for hHSF1 are represented by open boxes superimposed on sequences from all HSFs. Two regions of high conservation are identified in the shaded boxes C-1 and C-2. h = human; m = mouse; and ch = chicken.

and 443, subdomain II located between aa 464 and 489, and subdomain III located between aa 509 to 529. Contribution from regions of less importance (subdomains *a* and *b*) were also seen. Subdomain I was identified by comparison of constructs 5 and 11 where a drastic loss of activity resulted from deletion of 21 aa normally located immediately adjacent to HR-C in the context of the full length HSF. Subdomain II was revealed by the deletion of 25 aa in the central portion of the CTA1 (compare constructs 12 and 13). Removal of the C-terminal 20 aa also resulted in a large drop in activity (compare constructs 5 and 14), and this region was designated as subdomain III. When assayed individually in isolation, the major subdomains showed either no, or little activity. This phenomenon is illustrated by construct 16 for subdomain I, construct 21 for subdomain II, and construct 13 for subdomain III. The contributions of the minor subdomains is more context sensitive. The influence of subdomain *a* is seen in the differences in activities between constructs 11 and 12, and between constructs 16 and 17. Although subdomain *a* contributed positively to subdomains II and III in combination, and to subdomain I in isolation, it seems to have had little effect on subdomain II in isolation (compare constructs 19 and 20). Subdomain *b* contributed positively to subdomains I and II in combination (compare constructs 14 and 15) and, unlike subdomain *a*, to subdomain II in isolation (compare constructs 20 and 21). These results indicate that the entire region located C-terminal to HR-C functions as a

transcriptional activation domain (CTA1) with each of the subdomains making a synergistic contribution to the activity of the whole.

Point mutations were introduced into a portion of CTA1 in an attempt to identify individual aa residues critical to transcriptional activity. Since CTA1 seemed to be comprised of multiple elements, single aa changes were not likely to result in large changes in activity which would facilitate screening of mutants. To reduce this problem, only the C-terminal portion of CTA1 (construct 12) from aa residues 464 to 529 ($CTA_{464-529}$) was used for these studies. Although this fragment showed only 15% of intact CTA1 activity (Fig. 2), it is a highly conserved region with two major (II and III) and one minor (b) subdomains for transcriptional activity (Fig. 5 and 6). Analysis of point mutations within $CTA_{464-529}$ indicated the importance of conserved aa residues present in HSF1 and HSF2 families. Random mutations were introduced into $CTA_{464-529}$ of construct 12 using low fidelity PCR (Kassenbrock et al., 1993). Approximately 200 yeast transformants containing cloned DNA fragments derived from PCR mutagenesis were then assayed for β-gal activity. Roughly 15% showed impairment of activity ranging from 50 to 100%. The results of DNA sequence analysis of some of the clones with impaired activity are shown in Table 1. Mutations were assigned to two groups based on activity compared to the nonmutated $CTA_{464-529}$: Group A showing zero or slight residual activity, and group B exhibiting 30 to 50%

mutant	%-Gal	%-Lux /hGH	C-1	II	b	C-2	III
Group A:							
MA-1	0	6.9		**D477V**; D482A		**L515P**	P522K
MA-2	0			**D477V**; D482A		**L515P**	P521S
MA-3	<3	6.1	**A470V**		F500Y	L514P	
MA-4	<3			P489L		T516S	
Group B:							
MB-1	30-50				L491Q; E508G		
MB-2	30-50			S480P		**L515P**	K524term
MB-3	30-50	17.7			E508K		
MB-4	30-50	20	**A470V**				

Table 1. Analysis of PCR generated point mutations of $CTA_{464-529}$. Random mutations were placed in CTA1 domains II, b and III, selected in yeast. Four representative mutants were assayed in HeLa cells. Percentage β-gal and luciferase activities were determined relative to wild-type activity (construct 12) in yeast and HeLa cells respectively. C-1 and C-2 are regions of the CTA1 showing conservation among animal HSF1 and HSF2 families (Fig. 6). Mutations affecting amino acid residues showing conserved identity among HSF1 and HSF2 families are in bold letters, those affecting residues with conserved similarity are underlined.

activity. Mutations which resulted in partial loss of activity (Group B) were the most informative and revealed the importance of alanine 470 (mutant MB-4) and glutamic acid 508 (mutant MB-3). Alanine 470 shows identity among all members of the HSF1 and HSF2 families and is located within conserved region C-1 (Fig. 6). Amino acid residue 508 is adjacent to conserved region C-2. This position is occupied by negatively charged residues in vertebrate HSF1. The deleterious effect of removal of the negative charge at position 508 by mutations E508G and E508K, and the conservation of negative charge at position 509, suggests that both residues 508 and 509 may be required for C-2 function within the HSF1 family. Mutants MB-3 and MB-1 had roughly the same activity suggesting that mutation of L491 to Q (MB-1) had little effect. The A470V mutation within C-1 occurred twice demonstrating the critical role of alanine 470. The difference in activity between MB-4 and MA-3 indicates that either phenylalanine 500 or leucine 514 (within region C-2), or both, are also involved in transcriptional activation. In addition, the occurrence of the L515P mutation in three of the mutants suggests that this conserved aa residue of C-2 is also required for full activity. Assay of reporter activity of select mutations in HeLa cells showed similar results to the yeast expression studies with all mutant protein present at comparable levels (Fig. 7). It seems significant that in this limited

MA-1 MA-3 MB-3 MB-4 WT

Fig. 7. Western blot of point mutations of $CTA_{464-529}$. WT = wild type; Mutants from MA-1 to MB-4 were mutations selected from Table 1. All mutants were GAL4-DBD fusions and transiently expressed in HeLa cells. The expression of each mutants were measured by western blots using anti-GAL4-DBD antibody as probe.

analysis most changes involved either hydrophobic or negatively charged aa.

Attempts at Domain Mapping of Soybean HSF34 and HSF5 in Yeast

A strategy of deletion analysis similar to that used with hHSFs was used with soybean HSFs (GmHSFs) in order to identify domains involved in transcriptional activation and basal repression. In contrast to human HSFs, the full length soybean HSFs failed to show any activity when monitored using the GAL-*lacZ* reporter system (Table 2). To test whether the lack of activity was due to a failure to release basal repression, a series of N-terminal and C-terminal deletions were analyzed. As with the hHSFs, all constructs contained the GAL4-DBD fused to the N-terminus. No deletion was found for either GmHSF5 or GmHSF34 that resulted detectable β-gal activity (Table 2) suggesting that the transcriptional activation domains were not functional in yeast, or that activation domains were not present on these particular HSFs.

In the next series of experiments (Fig. 8), the acidic activation domain of GAL4 (aa 768 to 881) (Chevray and Nathans, 1992) was fused to the C-terminus of GmHSF34 to provide an activator known to be functional when fused to hHSF1 (Fig. 1, construct 8). As before, most constructs showed little or no activity under either control or hs conditions. However, removal of aa 103 through 154 from the N-terminus caused GmHSF34 to show 36 units of constitutive activity suggesting that this portion of the HSF was

construct	HSF	deleted amino acids	domains intact	activity (units)
22	GmHSF34	full length	D, HR-A, HR-B, HR-C	0
23	GmHSF34	DN 1-40	HR-A, HR-B, HR-C	0
24	GmHSF34	DN 1-102	HR-A, HR-B, HR-C	0
25	GmHSF34	DN 1-154	HR-A, HR-B, HR-C	0
26	GmHSF34	DN 1-175	HR-B, HR-C	0
27	GmHSF34	DN 1-203	HR-C	0
28	GmHSF34	DN 1-248	HR-C	0
29	GmHSF34	DC 250-282	D, HR-A, HR-B	0
30	GmHSF34	DC 205-282	D, HR-A, HR-B	0
31	GmHSF34	DC 156-282	D	0
32	GmHSF5	full length	D, HR-A, HR-B	0
33	GmHSF5	DC 349-370	D, HR-A, HR-B	0
34	GmHSF5	DC 329-370	D, HR-A, HR-B	0
35	GmHSF5	DC 309-370	D, HR-A, HR-B	0
36	GmHSF5	DN 1-255	-	0
37	GmHSF5	DN 1-255; DC 349-370	-	0
38	GmHSF5	DN 1-255; DC 329-370	-	0
39	GmHSF5	DN 1-255; DC 309-370	-	0

Table 2. Analysis of GmHSF-GAL4-DBD fusions in yeast cells. Fusion constructs of GmHSF34 and GmHSF5 were tested in strain PCY2 for β-gal activity under both hs (37°C) and control (25°C) conditions for 1 hr. No activity at either temperature was obtained for any of the constructs tested. N- and C-terminal deletions are denoted as DN and DC, respectively. The GmHSF34 protein is predicted to have 282 amino acids and GmHSF5 to have 370 amino acids. Domains: D, DNA binding domain; HR-A, hydrophobic repeat A of the OD; HR-B, hydrophobic repeat B of the OD; and HR-C, C-terminal hydrophobic repeat C. GmHSF5 has no identifiable HR-C. Fusion constructs 22 and 32 contained full length HSFs.

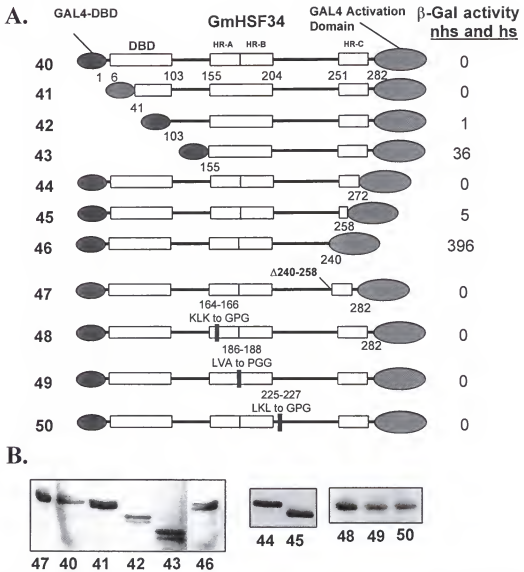


Fig. 8. Expression of GmHSF34-GAL4-DBD fusion proteins in yeast. A.) β-gal activity did not vary significantly between control (nhs) and heat shock (hs) conditions. All constructs were cloned in vector pYDA and expressed in yeast strain PCY2. Point mutations in constructs 48 through 50 were as follows: 164GPG (KLK 164-166 changed to GPG); 186PGG (LVA 186-188 changed to PGG); and 225GPG (LKL 225-227 changed to GPG). B.) Western blot of soybean HSF constructs expressed in yeast using anti-GAL4-DBD antibodies. Numbers correspond to constructs depicted in panel A. nhs, non-heat shock; hs, heat shock.

inhibiting activity of the GAL4 activation domain. When the C-terminal portion of HR-C was deleted, a low amount of constitutive activity was seen (construct 45). An even more dramatic increase in constitutive activity (396 units) was obtained by removal of 19 additional aa (aa 241 to 258) from the C-terminus suggesting that these 19 aa alone were responsible for the repression of activity. However, removal of these aa by internal deletion (construct 47) failed to activate transcription indicating that the entire HR-C must contribute to inhibition of the GAL4 activator. Substitution mutations within the OD and adjacent region were designed to disrupt the predicted coiled-coil structure by replacing hydrophobic residues and, perhaps, relieve basal repression. However, none of the three substitutions resulted in activity under either basal or heat shock conditions (constructs 47 through 50). Similar mutations in hHSF2 resulted in nuclear localization. The lack of effect of point mutations in the present study may have several interpretations and offers no obvious explanation for the lack of activity of the GmHSF34 constructs. Although these experiments did not identify domains involved in either basal repression or transcriptional activation, they did indicate that at least two regions of GmHSF34 are very inhibitory to the function of the acidic GAL4 activator domain: the linker region between the DBD (aa 103 to 154), and the HR-C region in the C-terminus (aa 249 to 282).

Substitution of scHSF with HSFs from Human and Soybean Cells

Human and soybean HSFs were substituted for the endogenous scHSF of yeast to assess the degree of conservation in the mechanisms of basal repression and transcriptional activation. Yeast strain YJB341 has a deletion in the chromosomal scHSF and is totally dependent for survival and normal growth on a copy of the scHSF present on a URA3 vector plasmid (YEpHSF^{RS}URA) (Bonner et al., 1992). The heterologous HSFs were introduced into YJB341 using plasmids pYGAL2 and pYDBD22 (VP16 fusion to C-terminal of HSF), and then selected on 5-fluoroorotic acid plates which eliminated the URA3 plasmid containing scHSF. Strain viability and HSF activity are shown in Fig. 9. HSF activity was monitored using the *HSE-lacZ* reporter gene present in yeast strain YJB341. The only nonviable substitution was with GmHSF34 (construct 54). This result was expected since no activity was seen when full length GmHSF34 was fused to the GAL4-DBD (Table 2, construct 22). Surprisingly, GmHSF5 was able to substitute for scHSF even though no activity could be detected in the GAL4 fusion system (Table 2; construct 32). No viable colonies were obtained using a deletion of GmHSF5 missing 42 aa on the C-terminus (construct 52) suggesting that the transcriptional activation domain may be located at the C-terminus. Viability of cells containing the C-terminal truncation was restored by the fusion of the VP16 acidic activator to the C-

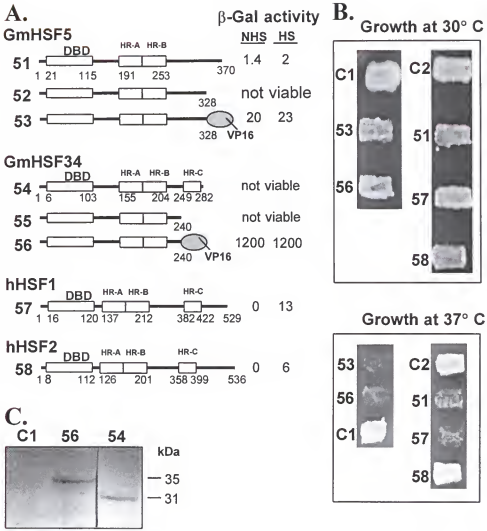


Fig. 9. Substitution of scHSF with HSFs from humans and soybean. A.) β-gal activity determined for all constructs in strain YJB341 after removal of scHSF by selection on media containing 5-FOA. Constructs 53 and 56 were derived from pYDBD22, all other were from pYGAL2. B.) Colonies after continuous growth on plates at indicated temperatures. Numbers indicate constructs shown in panel A. C1 and C2 were positive control strains containing either the heat inducible scHSF (strain YJB341), or constitutive scHSFM232V (341/BS16M232V), repectively. C.) Western blot of soybean GmHSF34 constructs 56 and 54 expressed in yeast using antiGmHSF34 antibodies. Due to the nonviability of GmHSF34 substitution, cells containing construct 54 were assayed before 5-FOA selection. Control C1 same as in panel B.

terminus (construct 53). In a similar manner, the fusion of the VP16 activator to a C-terminal truncation of GmHSF34 also resulted in viable cells (construct 56). In the case of the GmHSF34(ΔC-terminus)-VP16 fusion, β-gal activity was unusually high (1,200 units) compared to the analogous VP16 fusion with GmHSF5(ΔC-terminus) suggesting that remaining portions of GmHSF5 may be inhibitory. It should be noted that neither of the plant HSFs showed significant heat inducibility. One interpretation of these results is that the mechanism of basal repression for these two plant HSFs is not compatible with the yeast system. The lack of substitution in the case of GmHSF34 seems to indicate that the activation domain, if present, is also not compatible with yeast expression. An alternate interpretation is that these two soybean HSFs lack transcriptional activation domain and possess no inherent ability to regulate basal transcription. Both human HSF1 and 2 were able to substitute for yeast HSF (constructs 57 and 58) and exhibited basal repression and heat inducibility. As was the case in the GAL4-DBD fusion constructs, hHSF1 showed greater activity (β-gal) than hHSF2.

All HSF-substituted strains were able to sustain growth at 15°C, 30°C and 37°C, but all grew better at 30°C than at 15°C (not shown) or 37°C (Fig. 9). All strains, with the exception of hHSF2, were drastically inhibited at 37°C. The hHSF2-substituted strain grew almost as well as the wild-type yeast strain at this temperature. There was no correlation

between the activity of the HSF monitored by the β-gal assay and the ability to maintain sustained growth at high temperature.

Activity of hHSF1 and hHSF2 in Yeast under Sustained Heat Shock

The time course of activity for hHSFs in yeast lacking the endogenous HSF (scHSF) was determined by heat stressing cells at either 37°C (normal heat shock) or 40°C (severe heat stress) for up to 20 hr. The yeast control harbored the plasmid YEpHSF^{RS} URA3 (Bonner, 1991) which contained a C-terminal truncation of scHSF (at aa 583) and was heat inducible. Under heat shock temperatures the two hHSFs exhibited delayed activation detectable after approximately 30 min (Fig. 10). The yeast HSF showed a typical transient response (Sorger, 1990) with activity peaking from 1 to 2 hr after induction and declining to slightly less than one half of peak activity by 4 hr. In contrast, the hHSFs showed a gradual reduction in activity after a peak at 2 hr. After 20 hr at 37°C, scHSF had a residual activity of approximately 1 unit, hHSF2 had roughly 4 units, and hHSF1 still exhibited 10 units of activity. Yeast HSF was attenuated by 96%, whereas hHSF2 lost 64% of its activity, and hHSF1 was only reduced by 41%. Under severe heat stress (40°C), the transient nature of scHSF expression diminished. Peak values of scHSF activity after 2 hr were nearly identical to those at 37°C, but attenuation during extended periods of stress was impaired.

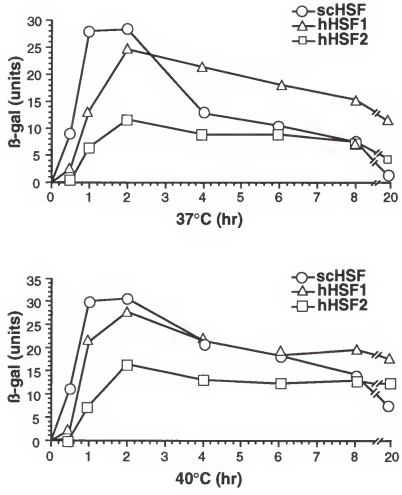


Fig. 10. Activity of hHSF1 and 2 under hs conditions in yeast cells lacking scHSF. Human HSFs were introduced into strain YJB341 using pYGAL2 vector and substituted for the endogenous HSF by selection on medium containing 5-FOA. The control strain was YJB341. HSF activity was monitored by assaying β-gal activity.

The hHSFs also showed less decline in activity under severe heat stress.

During prolonged heat stress, two noticeable differences were seen when hHSFs were substituted for the scHSF: a delayed activation, and the lack of a clearly defined transient response. The inability of the hHSFs to down modulate after 2 hr of heat shock may explain the poor growth at 37°C of yeast cells substituted with plant and human HSFs. However, this can not be the only reason since GmHSF5 showed relatively low activity (1 to 2 units, Fig. 9) under both control and heat shock conditions.

The Functional Targets of hHSF1 in Transcription

Strategies Used to Characterize Protein-Protein Interactions *in vitro* and *in vivo*

General assumption that underlies these experiments to characterize protein-protein interactions between HSFs and components of the PIC is that the predominant mechanism in transcriptional activation is based on recruitment mediated through direct protein-protein contacts. One strategy employed here to identify and map interactions *in vitro* entailed the binding of GTFs in solution to immobilized GST fusions consisting of various full length and deletion constructs of hHSFs (Fig. 11). The GST-HSF fusion proteins were bound to glutathione Sepharose beads forming an affinity matrix that was incubated with extracts containing

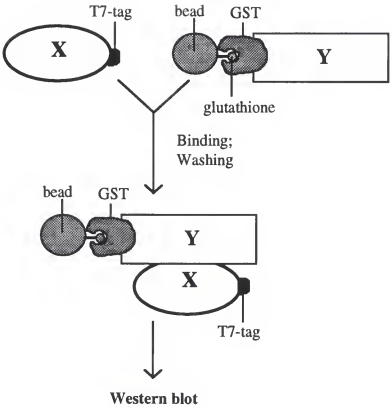


Fig. 11. GST-pull-down assay. Protein X is T7 epitope-tagged and protein Y is a GST fusion immobilized on glutathione Sepharose beads. After co-incubation, pelleting the beads will pull-down X if X and Y interact stably. Protein X released from the beads is visualized on western blot by probing with anti-T7 tag antibody. If protein X is not tagged, as in the case of endogenous factors from a HeLa nuclear extract, protein X-specific antibodies must be used in the western blot to determine interaction with protein Y.

recombinant peptides derived from GTFs expressed in *E. coli* or HeLa cells. The binding reactions were conducted at near physiological salt concentrations and pH to approximate conditions *in vivo*. Ligand proteins and their respective deletion constructs were T7-epitope-tagged to facilitate detection of bound proteins by western blotting. Negative controls included interactions with immobilized GST and non-binding mutants of scTBP(1-82) and VP16(Δ456-FP442). Wild type VP16(413-490) was also used as a positive control for binding where appropriate. The choice of factor proteins selected for testing was determined by availability and by previously demonstrated ability to interact with the acidic activator VP16.

Two approaches were employed to demonstrate interactions between hHSF1 and GTFs *in vivo*: co-immunoprecipitation of transiently expressed from HeLa whole cell extracts, and the squelch-rescue assay. In co-immunoprecipitation experiments a GAL4-DBD fusion construct containing either CTA1 or CTA1-Plus was co-expressed with a T7-tagged hTBP. GAL4-DBD fusions with the HSF activation domain fragments were used in order to provide an epitope for antibody precipitation, and to furnish a nuclear localization sequence (NLS). The GAL4-DBD fusion protein was immunoprecipitated on beads containing anti-GAL4-DBD antibody from whole cell extracts and the bound protein analyzed by western blots probed with anti-T7-epitope antibodies. The squelch-rescue approach will be discussed later.

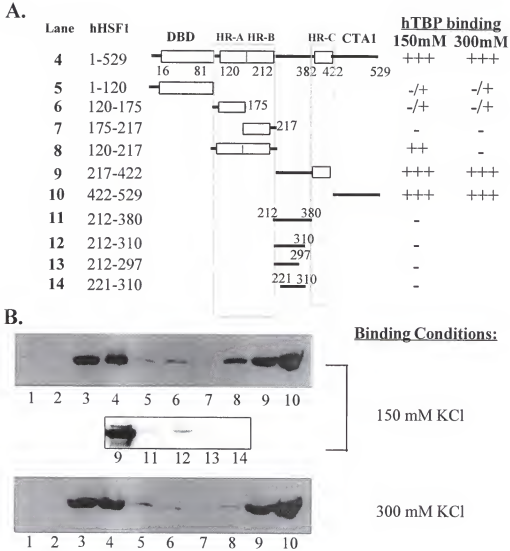


Fig. 12. Interactions between T7-hTBP and GST-hHSF1 fusions. A.) Summary of results of GST-pull-down assay at 150 mM and 300 mM KCl with T7-tagged hTBP expressed in *E. coli*. B.) Western blot of the bound hTBP probed with anti-T7-tag antibody. Lanes: 1 = GST (negative control); 2 = GST-VP16(Δ456-FP442) (negative control); 3 = GST-VP16(413-490) (positive control). All other lanes are GST-hHSF1 deletions represented in panel A.

In vitro and *in vivo* interactions between hHSF1 and hTBP

The GST-pull-down assay (Fig. 11) was used to map in detail the interactions between hTBP and hHSF1 *in vitro* (Fig. 12). Binding was conducted at moderate (150 mM KCl) and high (300 mM KCl). Strong binding at both salt concentrations was observed between full length hTBP (lane 4), the 217 to 422 construct (lane 9) and CTA1 (aa 422 to 529, lane 10). The interaction of hTBP with the 217 to 422 fragment was due to the presence of AD1 within HR-C, since removal of HR-C resulted in loss of binding (lanes 11 though 14). Weak binding was observed with protein fragments containing the N-terminus and DBD (aa 1 to 120, lane 5) and HR-A (lane 6). Moderate binding of hTBP occurred with a hHSF1 fragment containing the intact OD (aa 120 to 217, lane 8) at 150 mM KCl, but this interaction was significantly reduced at 300 mM KCl. In general, there was a correlation between the affinity of a fragment of hHSF1 for hTBP and its known involvement in transcriptional activation in that protein fragments containing either (AD1 construct 9), or AD 2 (construct 10), exhibited the strongest binding. The interactions seen with the DBD and the OD were weaker, and its biological relevance is not clear.

A similar series of experiments were conducted to map the region of hTBP that interacts with the transcriptional activation domains of hHSF1 (AD1 and AD2) present in the CTA1-Plus fragment (aa 382 to 529). In panel B of Fig. 13,

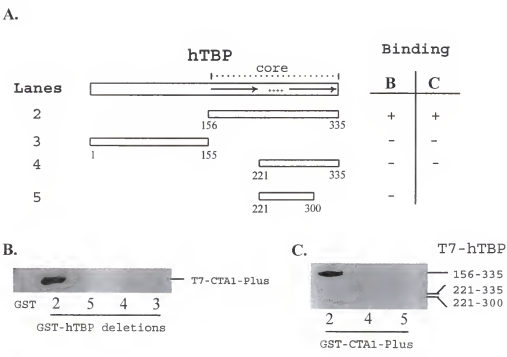


Fig. 13. *In vitro* interactions of hTBP deletions with CTA1-Plus. A.) Summary of interactions between hTBP and CTA1-Plus. Binding results are indicated for panels B and C. B.) Western blot of T7-tagged CTA1-Plus bound to GST-hTBP fusion proteins. T7-tagged CTA1-Plus from *E. coli* lysate was used in binding reactions with immobilized GST-hTBP fusion proteins. C.) Western blot of T7-tagged hTBP deletions bound to GST-CTA1-Plus. T7-tagged hTBP deletions were used to interact with GST-CTA1-Plus fusion protein and detected with anti-T7 tag antibody. Equal amounts of T7-tagged hTBP deletion proteins were added in each pull-down assay. The bars indicate the expected positions of bands representing deleted hTBP proteins. Lane numbers correspond to hTBP constructs in panel A.

matrix bound GST-fusion proteins consisting of various of deletion fragments of hTBP were incubated with T7-tagged CTA1-Plus. The region of hTBP responsible for the interaction corresponds to Repeat 1 of the core from aa 156 to 221. The reciprocal experiment using GST-CTA1-Plus as the matrix bound protein and T7-tagged deletion fragments of hTBP (expressed in HeLa cells) as the ligand gave identical results (Fig. 13, panel C). These results do not rule out the possibility that interactions between CTA1-Plus and hTBP may also involve core repeat 2 (C-terminal), since deletion of the N-terminal repeat may also disrupt conformation globally.

As a further test of the potential for interaction between the transcriptional activation domain of hHSF1 and hTBP, a GST-pull-down assay and co-immunoprecipitation experiments were conducted using proteins expressed in HeLa cells. In panel A of Fig. 14, immobilized GST-CTA1 was incubated with T7-tagged hTBP from whole cell HeLa extracts. CTA1 (AD2) was able to bind HeLa-expressed hTBP with high efficiency (compare input, lane 1, with lane 3). In the co-immunoprecipitation experiment, interactions were demonstrated between co-expressed GAL4-DBD-CTA1, or GAL4-DBD-CTA1-Plus and T7-hTBP (Fig. 14, panel B, lanes 4 and 5). A clear increase in binding was evident with the CTA1-Plus domain compared to CTA1. Significantly, the GAL4-DBD-CTA1 mutant (MA-1), was unable to bind hTBP. This lack of interaction is consistent with the drastically reduced

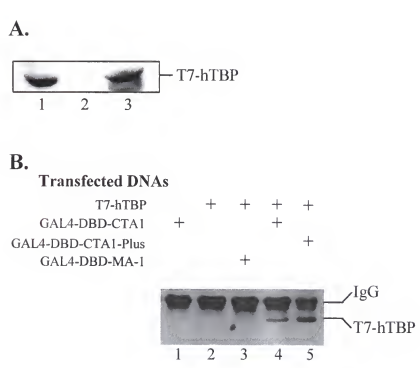


Fig. 14. Interactions between hTBP and hHSF1 demonstrated by co-immunoprecipitation. A.) GST-pull-down assay with whole cell extracts containing T7-tagged hTBP expressed in HeLa cells. Lanes: 1 = input; 2 = GST (negative control); 3 = GST-CTA1. B.) Co-immunoprecipitation of either CTA1, MA-1 or CTA1-Plus with T7-tagged hTBP co-expressed in HeLa. Plus sign (+) represents the presence of each plasmid in the transfection. Different combinations of plasmid DNAs were cotransfected into HeLa cells. Whole cell extracts were made and used for co-immunoprecipitation by anti-GAL4-DBD antibody covalently cross-linked to Protein-A-Sepharose beads. The co-immunoprecipitated T7-tagged hTBP was detected by probing the western blot with anti-T7-tag antibody. hTBP was T7-tagged in vector pNT7-SB; CTA1, CTA1-Plus (CTA1 plus HR-C) and MA-1 (a mutant of CTA464-529) were fused with the GAL4-DBD in vector pNG-SB.

transcriptional activity of the MA-1 mutant (Table 1) suggesting that CTA1-TBP interactions *in vivo* are essential for full activity.

Since hTBP is tightly associated with hTAFs in the TFIID complex *in vivo*, it is important to demonstrate that the activation domains of hHSFs are capable of interacting with the endogenous TFIID complex in addition to isolated TBP. In order to demonstrate that the GST-CTA1-Plus affinity matrix could bind TFIID in a HeLa nuclear extract, the pull-down assay was conducted and the membrane was probed with anti-TBP and then by anti-TAF250 (Fig. 15). GST-pull-down assays demonstrated that endogenous TBP in nuclear extracts was able to bind to the CTA1-Plus at salt concentration ranging from 100 mM to 250 mM KCl (Fig. 15A, lanes 3, 7 and 9). It was further demonstrated that the TBP bound to the GST-CTA1-Plus affinity matrix was incorporated in the TFIID complex since TAF250 was visualized upon reprobing the membrane with anti-TAF250 (Fig. 15B, lane 3). It is also noteworthy that the CTA1 domain was not able to bind the TFIID complex as efficiently as CTA1-Plus since ten-fold more extract and GST-CTA1 matrix were needed in order to detect the interaction (data not shown). This large difference in binding affinities may indicate synergism in recruitment of TFIID by the two activation domains (AD1/HR-C and AD2/CTA1); however, a simple additive effect in binding by CTA1-Plus can not be ruled out since the binding efficiency of isolated AD1/HR-C was not tested.

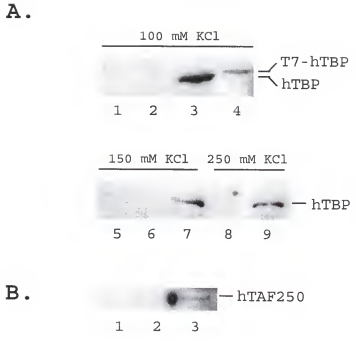


Fig. 15. hTFIID complex in HeLa nuclear extracts binds immobilized GST-CTA1-Plus in pull-down assay. A.) Western blot of hTBP in the hTFIID. 50 µl of nuclear extract and 20 µl of GST-CTA1-Plus were used for hTFIID complex pull-down assay at various salt concentrations. hTBP was visualized by anti-hTBP antibody. Lanes: 1, 5 = GST; 2, 6, 8 = GST-CTA1; 3, 7, 9 = GST-CTA1-Plus; 4 = T7-tagged hTBP expressed in *E. coli* as a size marker. B.) Western blot of hTAF250 in the hTFIID. The blot in panel A was reprobed with anti-hTAF250 antibody.

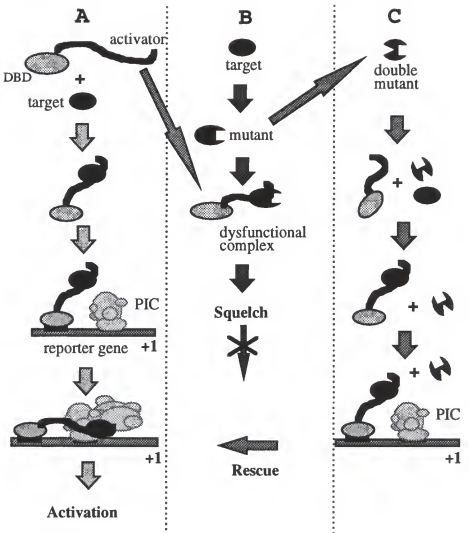


Fig. 16. The squelch and rescue assay. Column A: Expression of GAL4-DBD fusion with activation domain results in reporter gene expression when the target factor is recruited to the PIC by interaction with the activation domain. Column B: Co-expression of the GAL4-DBD/activator with a dysfunction mutant of the target factor results in formation of a dysfunctional complex which squelches transcriptional activity. Column C: Co-expression of the GAL4-DBD/activator with a double mutant of the target factor has little, or no effect on transcription if the second mutation removes the site of activator binding.

The squelch-rescue assay (Fig. 16) served as a final demonstration of protein-protein interactions *in vivo* between CTA1-Plus of hHSF1 and hTBP. In these experiments activity of the GAL4-DBD-CTA1-Plus activator protein was inhibited by co-expression of a hTBP mutant protein. A titration of effector vector conducted to insure that optimal expression levels of the activator (effector) were achieved that minimized the self-squelching effect of over-expression. As seen in Fig. 17, from 0.2 to 2 µg of the GAL4-DBD-CTA1-Plus plasmid DNA showed no significant self-squelching of activity. Transfection with 5 and 10 µg of the effector plasmid reduced activity by approximately 30 and 70%, respectively. These results indicated that transfection with 0.3 µg of the effector plasmid would be in the range of optimum expression and acceptable for use in subsequent squelch-rescue assays. When 0.3 µg of GAL4-DBD-CTA1-Plus plasmid was co-transfected with 3 µg of the hTBP (aa 156-335) mutant DNA, transcriptional activity was reduced by approximately 65% (Fig. 18B) consistent with the squelching of CTA1-Plus activity due to interaction between it and the hTBP fragment. Squelching was not seen when the CTA1-Plus effector was co-expressed with the hTBP (aa 221-335) mutant as predicted, since this hTBP fragment was shown by the GST-pull-down assay not to bind CTA1-Plus (Fig. 13). The reversal of squelching by the 221 to 335 aa fragment was not due to poor expression or stability of this protein as demonstrated by western blot analysis (Fig. 18A).

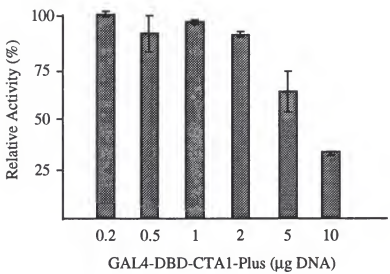


Fig. 17. Optimization of expression of the GAL4-DBD-CTA1-Plus effector in HeLa cells. Each transfection contained 0.2 to 10 µg of CTA1-Plus fusion in vector pNG-SB (GAL4-DBD-CTA1-Plus), 0.5 µg of growth hormone vector (pGXH5), 2 µg of luciferase reporter (pG5Luc) and appropriate amount of empty vector (pNeo-NB) added upto total 12.5 µg of DNA. Error bars were derived from three replicate experiments.

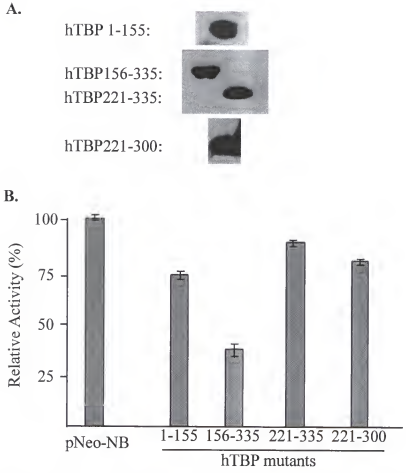


Fig. 18. *In vivo* interactions between CTA1-Plus and hTBP analyzed by the squelch-rescue assay. A.) Western blot of T7-tagged hTBP mutants transiently expressed in HeLa cells and detected with anti-T7-tag antibody. B.) Squelch-rescue assay of hTBP mutants demonstrating their effect on transcriptional activity of CTA1-Plus. The first effector was the CTA1-Plus fusion in vector pNG-SB (GAL4-DBD fusion vector). The second effector consisted of indicated hTBP deletions with the T7-tag and nuclear localization signal in vector pT7-NLS. The reporter was the luciferase gene with GAL4 DNA binding sites upstream of the TATA-box (pG5luc). pNeo-NB is a mammalian cell expression vector with only polylinker downstream of the CMV promoter (empty vector). The following DNAs were co-transfected into HeLa cells: 0.3 μg of GAL4-DBD-CTA1-Plus, 3 μg of hTBP mutant, 2 μg of pG5luc and 0.5 μg of growth hormone reporter (pXGH5). Luciferase activity of triplicate transfections was normalized using human growth hormone production to give relative activities.

Interactions between hHSF and hTFIIB

In vitro mapping of interactions between hHSF1 and hTFIIB revealed two regions of hHSF1 that show affinity for hTFIIB: the OD and a portion of the C-terminus (Fig. 19). As in the case with hTBP, interactions between hTFIIB and the OD were reproducibly strong, but difficult to interpret since this region can be deleted without affecting transcriptional activity. The other interaction at the C-terminus is potentially more significant due to the presence of activation domain AD1/HR-C (Fig. 19, lane 9). It appears that hTFIIB differs from hTBP by showing a strong preference for the AD1/HR-C, whereas hTBP interacted strongly with both AD1/HR-C and AD2/CTA1 (Fig. 12). It is also noteworthy that the region containing NR (aa 212 to 310) did not bind hTBP or hTFIIB *in vitro*. Although interacts with hHSF2 were not mapped in detail, it does have potential for hTBP interaction as demonstrated in Fig. 19, lane 2.

Results from the squelch-rescue assay were consistent with the occurrence of *in vivo* interactions between CTA1-Plus and hTFIIB. Substantial squelching of approximately 70% occurred with the hTFIIB$_{1-207}$ protein which includes the N-terminal domain and the first core repeat (Fig. 20). It is also significant that the hTFIIB$_{1-207}$ deletion containing a mutation (R185E, R193E) in helix E1 of repeat 1 showed a similar degree of squelching as the corresponding wild-type

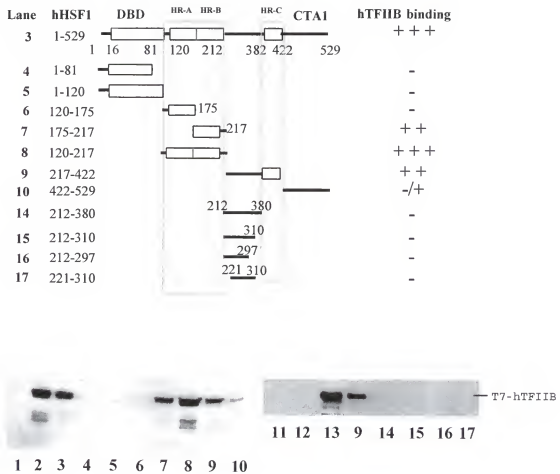


Fig. 19. Interactions between hTFIIB and hHSF1. Lanes: 1 = GST-scTBP(1-82) (negative control); 2 = GST-hHSF2; 11 = GST (negative control); 13 = GST-VP16(413-490) (positive control); Other lanes are GTS-hHSF1 deletions as indicated.

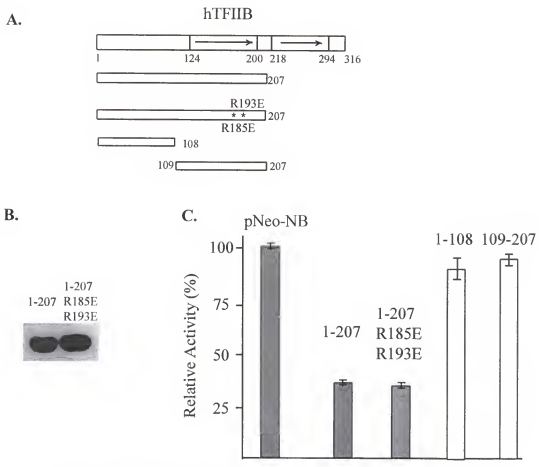


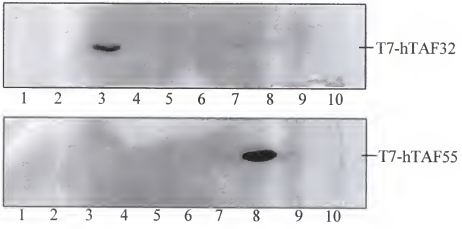
Fig. 20. Two fragments of hTFIIB squelch transcriptional activity of CTA1-Plus. A.) Schematic of hTFIIB and deletion mutants. The two astericks indicate two point mutations R185 to E185 and R193 to E193 in E1 helix of hTFIIB. B.) Western blot of T7-tagged hTFIIB mutants transiently expressed in HeLa cells and detected with anti-T7 tag antibody. C.) Squelching of CTA1-Plus activity by wild-type and mutated hTFIIB(1-207). The first effector was the GAL4-DBD-CTA1-Plus fusion in vector pNG-SB, the second effector was either of two hTFIIB deletion fragments with T7-tag and nuclear localization signal in vector pT7-NLS. The reporter was 2 μg of the luciferase gene with GAL4 DNA binding sites upstream of TATA-box. pNeo-NB is a mammalian cell expression vector with only polylinker downstream of CMV promoter (empty vector) 0.3 μg of GAL4DBD-CTA-Plus, 3 μg hTFIIB mutant, 2 μg of luciferase reporter (pG5luc) and 0.5 μg of growth hormone reporter (pXGH5) DNA were co-transfected into HeLa cells. The luciferase activity of each transfection triplicates was normalized by human growth hormone expression.

peptides. The failure of the helix E1 mutant to rescue the squelch of hTFIIB$_{1-207}$ indicates that CTA1-Plus, unlike VP16 (Roberts et al., 1993), does not require the E1 helix for binding.

Although squelching of CTA1-Plus activity relative to the growth hormone gene was clearly evident for hTFIIB$_{1-207}$ and hTFIIB$_{1-207}$ mutant, the conclusion that this attenuation was due to interactions between CTA1-Plus and hTFIIB was not as strong as in the case of hTBP since no reversal of squelching could be definitively demonstrated. Two constructs, hTFIIB$_{1-108}$ and hTFIIB$_{1-207}$, showed no squelching, but protein expression levels were to low to conclude that either of these constructs failed to interact with CTA1-Plus.

In vitro and *in vivo* interaction between hHSF1 and hTAF32, 55 and PC4

In addition to hTBP and hTFIIB, which have been shown to interact with multiple activation domains, three others less well characterized components of the PIC were screened for potential interaction with hHSFs. The first of these was hTAF32 which is a target for VP16 (Klemm et al., 1995) and therefore a potential target for AD1/HR-C (resemble an acidic activator). hTAF55 was chosen as a potential negative control since it does not bind VP16 (Chiang and Roeder, 1995), and PC4 was selected because it is required for efficient function of many types of transcriptional activators (Ge and Roeder, 1994). When immobilized protein



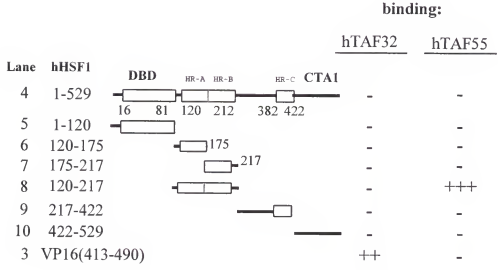


Fig. 21. Interactions of GST-hHSF1 with hTAF32 and hTAF55. Lanes: 1 = GST; 2 = GST-VP16(Δ456-FP442); 3 = VP16(413-490); Other lanes are GST-hHSF1 fusion proteins indicated.

A.

B.

Lanes	GST-fusions	PC4 binding
1	GST	-
2	GST-VP16(Δ456-FP442)	-
3	GST-VP16(413-490)	+++
4	GST-hHSF1	+/-
5	GST-hHSF2	+
6	GST-CTA1	-
7	GST-scTBP(1-82)	-
8	PC4 input	input

Fig. 22. Interactions of PC4 with GST-fusions. A.) Western blot of T7-tagged PC4 using anti-T7 tag antibody. B.) Summary of interactions between PC4 and hHSFs. Lanes 1, 2 and 7, negative controls; lane 3, positive control. Other lanes are GST-fusion proteins indicated.

fragments of hHSF1 were incubated with hTAF32 and hTAF55, no significant interactions were observed with the exception of hTAF55 binding to a fragment containing HR-A and HR-B (Fig. 21, lane 8). Again, it is hard to evaluate the significance of this interaction due to the lack of evidence that the OD contains a transcriptional activation motif. In these experiments GST-VP16 served as a positive control for binding to hTAF32 (Klemm et al., 1995) and a negative control for binding to hTAF55 (Chiang and Roeder, 1995). In the case of PC4, weak interactions were observed (Fig. 22, lane 5). Interactions with CTA1 were weak and interpreted as negative, since the faint band observed was no more intense than obtained using the VP16Δ456-FP442 (Fig. 18, lane 2), presumably a negative control. The weaker binding of CTA1 (AD2) relative to full length hHSF1 (Fig. 22, compare lanes 4 and 6) may indicate that AD2 is insufficient for optimum interactions and suggests that stronger binding may be possible using CTA1-Plus (AD1/HR-C plus AD2/CTA1). Other negative controls included GST alone (lane 1) and a fragment of yeast TBP, scTBP1-82. The VP16 protein positive control showed evidence of strong interaction indicating that the PC4 protein possessed binding activity under these conditions.

The VP16Δ456-FP442 mutant was used as a negative control for nonspecific binding based on its inability to interact with hTFIIB (Roberts and Green, 1994). However, its ability to bind hTAF32 and PC4 has not been previously characterized. The lack of binding to hTAF32 (Fig. 21, lane 2) and the

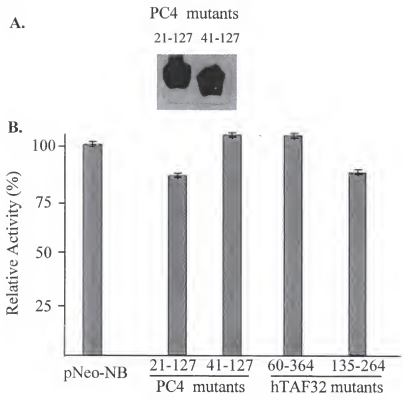


Fig. 23. A.) Western blot of T7-tagged PC4 and hTAF32 mutants. The T7-tagged PC4 and hTAF32 mutants were transiently expressed in HeLa cells, and detected with anti-T7 tag antibody. The expression of hTAF32 mutants were undectable. B.) Squelching and reversal of squelching effects of PC4 and hTAF32 mutants on the transcriptional activity of CTA1-Plus. The first effector was GAL4-DBD-CTA1-Plus fusion in vector pNG-SB, the second effector was PC4 or hTAF32 deletions with T7-tag and nuclear localization signal in vector pT7-NLS. The reporter was 2 μg of luciferase gene with GAL4 DNA binding sites upstream of the TATA-box. pNeo-NB is a mammalian cell expression vector with only polylinker downstream of the CMV promoter. 0.3 μg of GAL4DBD-CTA1-Plus, 3 μg of PC4 or hTAF32 mutant . 2 μg of luciferase reporter (pG5luc) and 0.5 μg of growth hormone reporter (pXGH5) were cotransfected into HeLa cells. The luciferase activity of each transfection was normalized by human growth hormone.

greatly reduced binding to PC4 (Fig. 22, lane 2) suggests that phenylalanine 442 of VP16 required for hTFIIB binding is also involved in interaction with hTAF32 and PC4.

Squelch-rescue assays using GAL4-DBD-CTA1-Plus confirmed *in vivo* the *in vitro* results which indicated that little or no interactions occur between hHSF1 and either hTAF32 or PC4 (Fig. 23B). A slight inhibition of CTA1-Plus activity was seen with the $PC4_{(21-127)}$ mutant, which was alleviated by removal of 20 aa at the N-terminus of this fragment. Western blot analysis (Fig. 23A) indicated (construct $PC4_{(41-127)}$) that the apparent result of squelch exhibited by $PC4_{(21-127)}$ was not due to lower levels of $PC4_{(21-127)}$ expression. Taken alone, the slight degree of squelching exhibited by $PC4_{(21-127)}$ seems consistent with the weak indication of interaction with hHSF1 and CTA1 obtained from the GST-pull-down assay (Fig. 22, lane 4). However, the level of squelch observed with $PC4_{(21-127)}$ is very similar to that obtained with hTAF32 (Fig. 23) which showed no indication *in vitro* of interaction (Fig. 21). For this reason, the slight squelch observed with $PC4_{(21-127)}$ must be interpreted with caution regarding the potential involvement of PC4 in hHSF1 activation.

Use of the Yeast Two-hybrid System to Screen for Proteins Interacting with hHSF1

In order to gain more insight into hHSF1-mediated transcriptional activation, the yeast two-hybrid system was used to screen a cDNA library of HeLa S3 cells in an attempt

to select out any relevant proteins interacting with hHSF1. Since hHSF1 has a transcriptional activation domain, a C-terminal truncation to aa 422 removing CTA1 of hHSF1 was engineered in order to use hHSF1 as a bait for screening. This strategy was resulted in selection of proteins that interacted with regions of hHSF1 other than CTA1. The residual activity of the bait vector alone (GAL4-DBD-hHSF1$_{(1-422)}$) was eliminated by addition of 20 mM 3-AT. Several clones were obtained including three independently selected clones encoding the β-subunit precursor of pyruvate dehydrogenase E1 component (Koike et al., 1990), single clones for phosphoprotein P1 (Koike et al., 1990) and ferritin (Dhar et al., 1993). Since there was no obvious links to hHSF1 or heat shock regulation for any of these selected proteins, this experimental approach was abandoned.

A Coupled GST-Pull-Down and Far-Western Analysis of Nuclear Proteins Capable of *in vitro* Interactions with the CTA1 of hHSF1

A novel technique combining the GST-pull-down approach (Fig. 11) with Far-Western analysis was developed to identify other potential targets of the CTA1 of hHSF1. In this procedure immobilized GST-CTA1 was used to bind proteins present in HeLa nuclear extracts prepared from hs and non-heat shocked cells. As a negative control for nonspecific binding, a fusion protein containing the negative regulator (NR, aa 212 to 310) was used in parallel at the GST-pull-down step (Fig. 24, lanes 4 and 5). Bound proteins were then

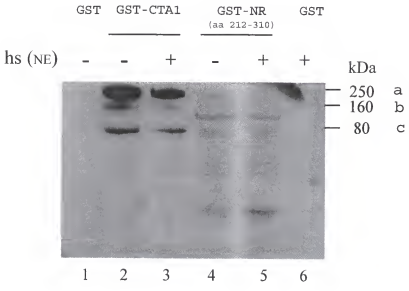


Fig. 24. Far-western blot of nuclear protein binding CTA1. Nuclear extracts were prepared from hs and non-heat shock HeLa cells and passed through either GST, GST-CTA1, or GST-NR affinity columns. Bound proteins were eluted, resolved by SDS-PAGE, membrane-blotted, and incubated with T7-tagged CTA1 protein. Protein-protein interactions were stabilized by chemical cross-linking and CTA1 containing complexes visualized by anti-T7-tag antibody. Lanes: 1 and 6, GST; 2 and 3, GST-CTA1; 4 and 5, GST-NR. NR is the negative regulation domain of hHSF1 (aa 212 to 310). The approximate molecular weight of proteins (a, b and c) bound to T7-CTA1 are indicated. NE, nuclear extract; hs, heat shock.

eluted with buffer A with 500 mM KCl and membrane blotted. The membrane immobilized proteins were then probed with T7-epitope-tagged CTA1 and the complexes stabilized by chemical cross-linking. CTA1 containing complexes were visualized by probing with anti-T7 epitope antibody (Fig. 24, lanes 2 and 3). Three major protein bands of approximately 250 kDa (*a*), 160 kDa (*b*) and 70 kDa (*c*) were detected in the GST-CTA1 pull-down experiment using extracts from non-heat shock cells (lane 2). Bands *a* and *c* were also pulled down from extract prepared from hs HeLa cells. The specificity of the interactions with CTA1 was demonstrated by the lack of a strong signal in the GST and GST-NR (negative regulator) (lanes 1 and 4-6). Although this experimental result suggests that proteins other than hTBP and hTFIIB potentially interact with CTA1, this interpretation was hard to verify due to a lack of reproducibility in subsequent experiments (two out of a total of four). A possible reason for variable results may have been due to inefficient renaturation of SDS denatured proteins immobilized on the membrane. These intriguing results were not followed through to a definitive conclusion due to time constraints and are included here to provide a complete experimental record.

Interaction between TFIID and Negative Regulation Domain

The GST-pull-down assay was used to detect possible interactions between TFIID and the negative regulation domain (NR) of hHSF1. Nuclear extracts prepared from hs and non-hs

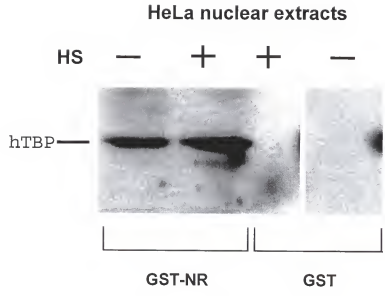


Fig. 25. hTFIID complex in HeLa nuclear extracts binds immobilzed GST-NR in pull-down assay. 500 µl of extract and 150 µl of GST-NR were used for hTFIID pull-down assays in 100 mM KCl salt. hTBP visualized by anti-hTBP antibody. Legends: GST-NR, GST fusion of the negative regulator domain of hHSF1; GST, a negative control; HS, heat shock treatment.

HeLa cells were incubated with either GST-NR or GST (as a negative control) at 100 mM KCl. Proteins bound to the affinity matrices were blotted and probed with anti-TBP antibody. The presence of TBP is evident in the pull-down assay using GST-NR, and the specificity of the interaction is demonstrated by the lacks of binding to GST. It is also significant that similar amounts of TBP exist from hs and non-hs nuclear extracts (Fig. 25). Because the inability of NR to interact with TBP directly (Fig. 12), TBP is assumed to interact indirectly through tight association with other proteins that contact the NR. The most likely candidate for these bridging proteins are the TAFs (one or more).

Repressor Function of the Negative Regulation Domain

Previous studies (Green et al., 1995; Newton et al., 1996) indicated that the NR does not have transcriptional activity when fused with GAL4-DBD, but has inhibitory effects on activators when it is directly fused with these activators. In order to understand the mechanism of the NR-mediated repression, the full NR and two deletions were fused with the GAL4-DBD and transiently co-expressed with GAL4-DBD-CTA1-Plus effector in HeLa cells. CTA1-Plus activity was monitored using luciferase/human growth hormone reporters. Equal amounts of GAL4-DBD-CTA1-Plus were co-transfected with a second effector plasmid containing either full length or deleted NR constructs, or the empty vector pNeo-NB. Repression (active repression) in this assay is defined as a

reduction in transcriptional activity greater than that predicted to occur from the presence of a neutral peptide fused to the GAL4-DBD. For example, if no repression occurred when equal amounts of GAL4-DBD-CTA1-Plus and GAL4-DBD-NR were co-transfected, the activity should have been around 50% compared with the empty vector control. This reduction in activity (passive repression) would result from formations of GAL4-DBD-NR homodimers (with no activity) and GAL4-DBD-NR/CTA1-Plus heterodimers (with half the activity of GAL4-DBD-CTA1-Plus homodimers). However, if the NR actively repressed transcription by blocking transcription in *trans* by poisoning the heterodimer and inhibiting neighboring GAL4-DBD-CTA1-Plus homodimers, reporter activity would be significantly less that 50%. The results presented in Fig. 26 indicate that the full length NR(212-310) reduced CTA1-Plus activity by more than 50% suggesting that the NR activity inhibited transcription in *trans*. Active repression was also exhibited by the NR(221-310) construct, but not by a C-terminal truncation of the NR (Fig. 26).

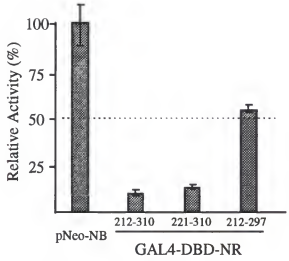


Fig. 26. Negative efffects of NR on the activity of CTA1-Plus. GAL4-DBD-CTA1-Plus and GAL4-DBD-NRs were coexpressed in HeLa cells using vector pNG-SB. Each transfection contained equal amounts of GAL4-DBD-CTA1-Plus DNA (0.5 μg) and either GAL4-DBD-NR constructs (0.5 μg), or pNeo-NB (0.5 μg) as the empty vector control. 0.5 μg of growth hormone vector (pXGH5) and 2 μg of reporter plasmid (pG5Luc) were included in each transfection. Relative luciferase activities were normalized using growth hormone production. Error bars were derived from three replicate experiments.

DISCUSSION

The human and plant HSFs evaluated in this study showed clear differences in activity when expressed in yeast. hHSF1 and 2 were active, and hHSF1 was heat inducible, and both were able to substitute for the endogenous yeast HSF. In contrast, soybean GmHSF34 and 5 were inactive under all conditions tested in yeast with the possible exception of GmHSF5 which was able to substitute for yeast HSF.

Overall, the degree of conservation in the mechanisms of HSF function between yeast and human cells was adequate to support growth of yeast cells when yeast HSF was substituted by hHSF1 or hHSF2. However, several aspects of HSF1 regulation were different in yeast cells when compared to HeLa expression. Examples of these difference include the degree of involvement of HR-A in basal repression, activity of HSF2, and the rate of hHSF attenuation after a period of heat stress.

Mechanism of Basal Repression

The strong activity of the isolated C-terminal portion of hHSF1 (AD1 and AD2) under non-hs conditions is consistent with the constitutive activity shown by the isolated C-terminal transcriptional activators (CTAs) of *S. cerevisiae* and *K. lactis* HSFs when fused to heterologous DBDs (Jakobsen

and Pelham, 1991; Nieto-Sotelo et al., 1990; Sorger, 1990). The inherent activity of the hHSF1 C-terminal domain is illustrated by the progressive increase in activity under non-hs conditions as aa residues N-terminal to the activation domains were sequentially removed (Fig. 1, constructs 1-4). Activity under basal conditions suggests that no stress specific modifications of the activation domains are required during hs expression, and lends support to models of basal repression based on masking of the HSF. These results do not, however, rule out the possibility that the activation domains must be modified posttranscriptionally; only that potential modifications are not stress specific.

Masking models of basal repression propose that activity of the various functional domains of HSFs are inhibited due to their inaccessibility during non-stress conditions (for review see (Sorger, 1991)). Repression is thought to result from the conformational state of the HSF itself, or by interactions of the HSF with other proteins. Hydrophobic repeats located within the OD and HR-C, and a region within AD2 have been shown to play a role in maintaining basal repression (Jakobsen and Pelham, 1991; Nieto-Sotelo et al., 1990; Rabindran et al., 1993; Sheldon and Kingston, 1993). One model for basal repression involves folding of the HSF under non-stress conditions so that HR-C interacts with one, or more, hydrophobic repeats of the OD (Nakai and Morimoto, 1993; Rabindran et al., 1993; Sheldon and Kingston, 1993). Our experiments do not distinguish between inter- and

intramolecular masking, but indicate that several regions of the hHSF1 protein contribute to repression of activity under both basal and hs conditions. For example, removal of HR-A of the OD (aa residues 120 to 174) caused activity under hs conditions to increase by 3-fold, and resulted in a significant loss of basal repression in yeast and a slight loss of basal repression in HeLa cells (Fig. 1). The large differences in activity between the isolated activation domains (constructs 4 and 5) and the full length protein (construct 1) indicates that a substantial amount of inhibitory potential exists in the full length protein, even during hs.

The loss of basal repression resulting from the removal of HR-A is consistent with the study of Sheldon and Kingston (Sheldon and Kingston, 1993) where point mutations introduced into either HR-A or HR-B resulted in constitutive nuclear localization of hHSF2. The involvement of HR-A in the regulation of basal activity in hHSF 1 and 2 differs from *K. lactis* HSF where only HR-B participates in the repression of activity (Chen et al., 1993). In Fig. 1, a correlation seems to exist between basal repression of hHSF1 and the presence of an intact OD. In these experiments DNA binding was conferred by the GAL4-DBD and is presumably not dependent on the oligomerization state of the HSF as indicated by the binding of construct 2 to the GAL4 DNA binding site under basal conditions (Fig. 3). The clear accessibility of the GAL4-DBD to DNA under non-hs conditions suggests that the

masking thought to be responsible for basal repression does not directly involve the DBD. Since DNA binding by the native hHSF1 can be inhibited indirectly by the prevention of trimerization, there is no requirement to physically block DNA access under conditions of basal repression.

Negative Regulation of Transcriptional Activation Domains

The lack of AD1/HR-C function after removal of AD2/CTA1 by C-terminal deletion (Fig. 1, construct 6) was unexpected since similar experiments by Green *et al.* (Green et al., 1995) and Newton *et al.* (Newton et al., 1996) showed from 5- to 11-fold heat induction. In those studies the OD was not present in the GAL4- or LexA-DBD fusions; whereas, in our construct 6 the entire protein was present except for AD2/CTA1, normally at the C-terminus. In the previous studies the 201 to 370 aa fragment exhibited heat inducible activity in HeLa and non-regulated, high activity in yeast (LexA-DBD fusion). The lack of AD1/HR-C activity in our experiments may indicate that negative regulation is in effect under all conditions, with a defect in mechanism for the release of negative control under hs conditions. This failure to release repression during heat stress may be related to the presence of the OD which has been associated with negative regulation in previous studies (Zuo et al., 1995) and by the results shown in Fig. 1. Another consideration is the possibility that the presence of LexA

and GAL4-DBDs may have differential effects on expression in the yeast system.

HSF2 Expression

GAL4-DBD fusions with hHSF2 were heat inducible in yeast, but showed little activity in HeLa cells (Fig. 1). In addition, the isolated C-terminus (aa 397 to 536) exhibited activities from 10 to 11% of the isolated HSF1 AD2/CTA1 in yeast under both basal and hs conditions. In contrast, very little activity was obtained in HeLa with the full length HSF2 (construct 9) and the isolated C-terminal domain (construct 10) showed less than 2% of the activity of the isolated AD2/CTA1. Furthermore, a decrease in the residual activity of the C-terminus was observed after hs. This striking difference in expression of HSF2 in yeast and HeLa cells seems to imply that different mechanisms of regulation are utilized in these two organisms suggesting the possibility that a developmentally-specific regulatory pathway may be involved in human cells that is absent in yeast. It is noteworthy that HSF2 is predominately involved in non-stress-induced Hsp gene expression during development and is active in hemin-induced differentiation of mouse embryonic carcinoma cells, in heart and brain cells, and during specific stages of spermatogenesis (Goodson et al., 1995; Sarge et al., 1994; Sistonen et al., 1992).

The co-expression of GAL4-DBD fusions of CTA1 and CTA2 resulted in non-synergistic activation of the reporter gene

in HeLa (Fig. 4). This result suggests that the synergistic response in Hsp70 expression seen previously (Sistonen et al., 1994) in human K562 cells that were induced with both hemin and heat stress was not due synergism between transcriptional activation domains of HSF1 and HSF2. In our studies we have assumed that opportunities for synergistic interactions between activation domains of HSF1 and HSF2 were provided by the formation of both homo- and heterodimers of the GAL4-DBD fusion proteins and by the occupancy of mixtures of heterodimers and homodimers (two types) bound to the same promoter. In view of the lack of synergism between HSF1 and HSF2 activation domains, it seems likely that co-expression of endogenous hHSF1 and hHSF2 facilitates the binding of HSF1 to hs promoters. It has been reported that during hs HSF binds to over 150 sites scattered over the genome of *Drosophila* that are non-hs genes (Westwood et al., 1991). It is also known that HSF1 binding to HSE elements is much more cooperative than HSF2 binding (Kroeger and Morimoto, 1994). Perhaps the non-hs sites are preferentially occupied by HSF2 during conditions of co-expression making more HSF1 available for synergistic binding to hs promoters.

Activation Domains in HSFs

The striking difference in activities of the human HSFs and soybean HSFs in yeast cells may be interpreted in two ways: either transcriptional activation domains of mammals and plant HSFs may be different in their compatibility with

yeast, or the soybean HSFs tested in this study lack efficient activation domains. Although a number of HSF activation domains have not been mapped in detail, it appears that most possess an overall negative charge and are enriched for serines. The *Kluyveromyces lactis* HSF (klHSF) activation domain (aa 592 to 623) is comprised of 22% negative aa residues, the activation domain of *Saccharomyces cerevisiae* HSF 16% (aa 628 to 671), and CTA1 of hHSF1 has 13% negative residues (Chen et al., 1993; Rabindran et al., 1991). Serines represent from 12 to 24% of the aa residues in scHSF and hHSF1, respectively, but are only 3% of the klHSF activation domain. The aa composition comparison between CTA1 and proline-rich activation domains indicates that CTA1 should be designated as a proline-rich activator (Table 3) (Artandi et al., 1995).

In higher plant HSFs, only the tomato transcriptional activation domains have been characterized (Treuter et al., 1993). Although the three tomato HSFs contain a concentration of negative charge in the C-terminal portion of the proteins (Treuter et al., 1993), several soybean HSFs do not. The C-terminal region of soybean GmHSF5 is nearly neutral, whereas GmHSF34 is basic.

A high degree of conservation is present in the primary aa sequence of the C-terminus of HSF1 and 2 in humans and mouse cells, and HSFs 3a in chickens (Nakai and Morimoto, 1993; Rabindran et al., 1991; Sarge et al., 1991; Schuetz et al., 1991). A comparison of C-terminal aa between six

	TFE3 134aa	**CTF1** 102aa	**Oct-2** 146aa	**AP2** 70aa	**CTA1** 108aa
Pro	13	19	13	21	18
Ser	16	13	14	9	24
Leu	13	10	8	9	13
Gly	9	10	10	7	8
Acidic	15	5	4		13

Table 3. Comparison of amino acid residue composition (%) of proline-rich activation domains (Artandi *et al.* 1995) with CTA1 domain of hHSF1.

vertebrate HSFs and AD2/CTA1 subdomains of hHSF1 mapped in yeast reveals two blocks of conserved aa (C-1 and C-2, Fig. 6) which show a general correlation with the location of subdomains II and III. This is especially true for subdomain II where 50% of the aa are either identical or similar between HSF1 and HSF2 classes. Even though searches of available protein data bases failed to show matches with the consensus for subdomain II in other proteins, there is little to suggest that this region may be exclusively specialized for HSF function since heterologous activator domains were able to replace AD2/CTA1 of hHSF1 without impairing heat inducible activation of hHSF in yeast cells (Fig. 1).

One of the best characterized acidic activation domains is from the herpes simplex virion protein VP16. It maps to the C-terminal 78 aa residues and can be divided into two subdomains consisting of hydrophobic residues embedded within regions of negative charge. The N-terminal subdomain contains phenylalanine residue 442 (Phe-442) at its core and the C-terminal region includes Phe-473 and Phe-475. Although AD2/CTA1 resembles a proline-rich activator in term of amino acid composition (see detailed discussion in the section, Attributes of Activation Domain, Literature Review), it also has regions that are similar to acidic activators. AD2/CTA1 contains four phenylalanines, most of which are located near negatively charged or bulky hydrophobic residues in configurations that are roughly similar to that found in VP16.

Plant HSFs contain a distinct activation motif designated as the TRP element which in tomato HSFs closely resembles the N-subdomain of VP16. Like VP16, the TRP element contains an aromatic residue (tryptophan instead of phenylalanine) located in a short region of negative charge (Treuter et al., 1993) and bulky hydrophobic residues. All three of the tomato HSFs and *Arabidopsis* HSF1 (atHSF1) contain at least one copy of this motif, but some soybean HSFs do not have recognizable TRP elements. The two soybean HSFs analyzed in this study contain one putative TRP element each. It is not known if the low activity observed is inherent to these particular HSFs, or simply reflects the inefficient function of this domain in yeast.

The TRP elements in tomato HSFs appear to be responsible for basal as well as heat inducible activity (Treuter et al., 1993). Transient assays in tobacco protoplasts indicate that all three tomato HSFs possess considerable amounts of basal activity. For full length tomato HSFs, hs causes transcription to increase from 1.6 to 4.4-fold. Removal of the C-terminal portion (76 aa residues) of tomato HSF8 deletes one of the two TRP elements present and results in a sharp reduction in basal activity, but has little effect on heat inducible activity. Further deletion removing the second TRP element causes in a loss of heat inducibility as well. The activation domains of tomato HSF30 and HSF24 are also C-terminal, but regions responsible for basal and heat inducible activity more closely coincide.

In each case, transcriptional activity (basal or heat inducible) is closely correlated with the presence of one or more TRP elements. In the case of tomato HSF8 and HSF30, one of the TRP elements is present within HR-C. Although both human and tomato HSFs appear to rely on acidic activation motifs that are similar to the transcriptional activation domains of VP16, soybean HSF34 and HSF5 activation motifs have not been identified in this study.

Recent experiments by Dr. Eva Czarnecka-Verner (unpublished) indicated that soybean HSF34 and HSF5 have no activity when examined by transient expression assays in a variety of plants and plant tissues. These results suggest that the lack of transcriptional activity exhibited by the soybean HSFs in yeast cells was not due to an incompatibility in the function of activation motifs between plants HSFs and yeast, but most likely reflects the absence of activation domains in GmHSF34 and GmHSF5. A potential caveat is the ability of GmHSF5 to substitute for yeast HSF which may be due to a cryptic activation domain of very low activity present in GmHSF5 that resulted in activity that was below detection levels in liquid assays of β-gal, but sufficient for growth of yeast cells under normal condition.

Mechanism of Transcriptional Activation of hHSF1

In *Drosophila*, transcription factors including dHSF, TFIID and pol II are pre-bound to Hsp70 gene promoters and transcription has been initiated waiting for productive

elongation before hs. Pol II was shown to be paused at a position 21-35 nucleotides downstream of the transcription start site by nuclear run-on analysis and *in vivo* footprinting in *Drosophila* Hsp70 genes (Giardina et al., 1992; Rasmussen and Lis, 1995). Pausing of pol II was also observed in nucleosome-packed human Hsp70 gene *in vitro*. Activators such as isolated activation domain of hHSF1 as well as SWI/SNF can inhibit the pausing and stimulate the readthrough of pol II in nucleosome-containing promoter of human Hsp70 gene when assayed with *in vitro* transcription system (Brown et al., 1996). It is suggested that activators can decrease the nucleosome-mediated inhibitory effect on transcriptional elongation resulting in the suppression of the pausing (Brown et al., 1996).

In the absence of the GAGA factor, the *Drosophila* Hsp70 promoter is not sufficiently accessible in chromatin, for dTFIID to bind to TATA-box (Shopland et al., 1995). The GAGA factor is a constitutively expressed transcription factor in *Drosophila*, and binds to GA/CT rich elements called GAGA elements. The binding of the GAGA factor to DNA opens the chromatin and makes DNA promoter accessible for HSFs, dTFIID, presumably other transcription factors. The GAGA factor, along with TATA box and initiator element, can stimulate the pausing and the level of non-productive transcription, and dHSF has no effect on transcriptional elongation and pausing in the same context when GAGA factor is absent (Lee et al.,

1992). However, the presence of both factors stimulates the high level of productive transcription (Lee et al., 1992).

HSF may have direct effect on processivity of transcriptional elongation. Alternative explanations are that HSF-mediated recruitment of GTFs, under hs, overrides the pausing and pushes pol II through pausing sites to generate non-abortive transcripts, or the GAGA factor-facilitated pausing is heat labile. Regardless the exact mechanism for HSF-facilitated stimulation of transcriptional elongation, the simplest explanation is that the phenomenon observed in *Drosophila* is limited to a small number of genes with unique promoter structure such as having GAGA elements, pausing sites, etc. This explanation is supported by the observation that the region of upstream of the *Drosophila* Hsp70 TATA box, including GAGA elements and HSEs, can program the formation of a paused pol II on a non-hs gene promoter with no detectable pausing normally (Lee et al., 1992). It is also reinforced by mutation analysis of GAGA element and HSEs: mutation of GAGA element reduces the pausing, and mutations of HSEs have no effects on pausing (Lee et al., 1992). Therefore, the GAGA-mediated pausing and HSF-facilitated stimulation of transcriptional elongation are not HSF-specific, nor hs-response specific; they are GAGA factor and GAGA-element specific. In other words, the main stream of HSF-mediated transcriptional activation is initiation instead of elongation or pausing. Moreover, the pausing under non-hs and quick release of paused pol II under hs in

Drosophila Hsp70 genes only account for the first round of transcription, thereafter, the transcriptional initiation of these genes will become the key step of regulation by HSFs.

The Role of the Interaction between hTBP and hHSF1

TBP contains determinants for protein-protein interactions with pol II, some of the TAFs and discrete sets of activators and repressors (Tang et al., 1996). TFIID or TBP binding to chromatin templates is rate limiting for gene expression, and the kinetics of this process is subject to regulation by activators *in vivo* (Klages and Strubin, 1995; Klein and Struhl, 1994). Two recently published papers (Moqtaderi et al., 1996; Walker et al., 1996) indicated that in yeast activated transcription *in vivo* generally does not require TAFs. TBP and TFIIB can be the direct target of transcription activators. Results from the *in vitro* and *in vivo* studies described here strongly suggested that TBP and/or TFIID is one of the direct target of HSF which leads to transcriptional activation of hs genes. hHSF1 can function at least through facilitation of hTBP or TFIID binding to the TATA box and thereby facilitate formation of the PIC. There is strong indication that TFIIB is also involved the pathway of HSF1 activation of transcription. These studies also indicate that hTAF32 and hTAF55, as well as PC4, are not the direct target for transcriptional activation by hHSF1.

In addition to clear evidence for TBP and TFIIB having important roles in HSF1-activated transcription, preliminary evidence from coupled GST-pull-down/Far-Western analysises (Fig. 24) suggested that other nuclear proteins may also be the direct targets of hHSF1 and involved in transcription regulation of hs genes.

Model for Heat Inducible Regulation of HSF

The results of binding studies indicate that two regions of hHSF1 can form complexes with components of TFIID: activation domains (CTA1-Plus) with hTBP, and the NR with a protein(s) associated with hTBP, probably a TAF(s). The ability of an activator protein to bind a GTFs is generally thought to result in recruitment of the GTF to the PIC and result in transcriptional activation. This picture of activator function is consistent with the correlation in TBP/TFIID binding by CTA1 and CTA1-Plus and their involvement in transcriptional activity. In contrast, the binding of the NR to TFIID under hs and non-hs conditions lacks any correlation between binding and transcriptional activity. These results and the strong *trans* repression of CTA1-Plus activity when CTA1-Plus and NR are co-expressed as GAL4-DBD fusions suggests that the NR/TFIID complex is dysfunctional with regard to transcription.

A model for transcriptional regulation of HSF1 is presented in Fig. 27. At normal temperatures, the activation domains are masked and inaccessible for the

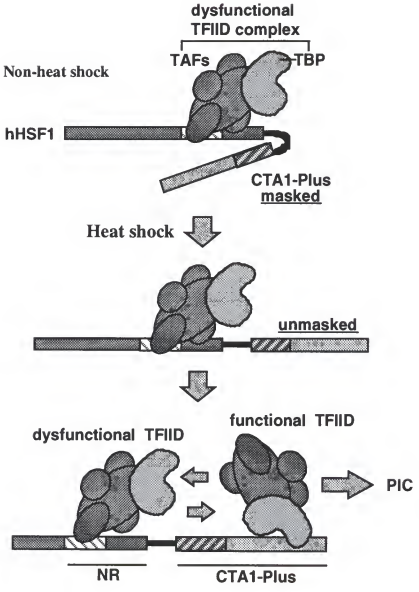


Fig. 27. Model for heat inducible regulation of hHSF1. Legend: CTA1-Plus, activation domains (AD1 and AD2) of hHSF1; NR, negative regulation domain; TFIID, a complex of TBP and TAFs. PIC, pre-initiation complex. Under basal conditions affinity for TFIID is mediated by TAF interaction with the NR resulting in a dysfunctional complex. After hs the C-terminal activation domains are unmasked and accessible for interaction with TBP and TFIIB. TFIID is then in equilibrium between the NR and CTA1-Plus. Binding to CTA1-Plus facilitates formation of the PIC.

formation of the functional TFIID complex on promoters; therefore, no activation occurs. The NR is capable of association with TFIID, presumably with one of the TAFs, forming a dysfunctional complex and leading to no-activation under non-hs conditions and inactivation of transcription during attenuation of the hs response, or upon removal of hs. According to the model, hs unmasks the activation domains resulting in the formation of functional TFIID complex and efficient recruitment of the TFIID to the promoter leading to transcriptional activation. The equilibrium between the dysfunctional TFIID (bound to the NR) and the functional TFIID complex (bound to the activators) is influenced by the conformational changes of HSFs under stress conditions. This model can explain the prompt and massive hs response by the rapid shifting of the equilibrium. This model is also consistent with the observation that the transcriptional activity of the activation domains in full length context can not be fully released even under hs conditions (Fig. 1). Only partial release of the activity under hs can also be observed in published studies (Green et al., 1995; Newton et al., 1996). For example, the levels of the released activities (hs conditions), range from 27% to 100%, and seem to depend on the activation domains located adjacent to the NR. When the activation domain is HR-C (aa 371-430), only 27% of the activity released under hs; when AD1 (aa 401-420) present, 83% released; when VP16 (aa 413-456) fused, 59%

released; when VP16 (aa 452-490) exists, full release obtained (Newton et al., 1996).

Basis for Future Studies in Plants

Information regarding hHSF targets of contact and the model of negative regulation of hHSF1 will serve as a starting point in future studies to characterize the modes of transcriptional regulation employed by plant HSFs. For example, since human HSFs have been shown to function in tobacco protoplasts (Treuter et al., 1993), it is reasonable to expect that plant HSFs may also target TBP and TFIIB for activation. The question of negative regulation of plant HSFs is still very much open, since no heat-inducible intrapeptide negative regulation domain have been identified. However, the C-terminal domains of GmHSF34 and GmHSF5 were able to repress transcriptional activation domains, GAL4 and VP16 (Fig. 8 and 9). These results indicate that plant HSFs, especially those lacking transcriptional activation domains, may form dysfunctional complexes in a manner analogous to the NR/TFIID association postulated for hHSF1. Such complexes may, or may not involve TAFs, since no TAFs have been cloned from plants and their role in activated transcription is unknown in plants.

SUMMARY AND CONCLUSIONS

Transcriptional activation domains were mapped in yeast to the C-termini of hHSF1 and hHSF2. Detailed mutagenesis of CTA1 identified multiple subdomains which make positive and synergistic contributions to activity. Analysis of point mutations in CTA1 revealed the importance of hydrophobic and charged amino acid residues for the transcriptional activity. Although co-induction of hHSF1 and hHSF2 *in vivo* has been reported to result in a synergistic increase in transcription of Hsp70 gene (Sistonen et al., 1994), the isolated CTA1 and CTA2 domains were not able to synergistically activate transcription in *trans* when co-expressed as GAL4-DBD fusions in HeLa cells.

For hHSF1, removal of HR-A caused a loss of basal repression when assayed in yeast, but not in HeLa indicating a lack of complete compatibility in mechanism between these two organsims. Although yeast was able to survive when its endogenous HSF was functionally substituted with either hHSF1 or hHSF2, differences in the hs response between yeast and HeLa cells were evident in the 30min delay in HSF activation after hs and the reduced ability to attenuate HSF activity after a transient hs.

GAL4-DBD fusions of hHSF1 and hHSF2 were transcriptionally activated in yeast cells in response to

heat stress. In contrast, neither of the soybean GAL4-DBD-HSF fusions (GmHSF34 and GmHSF5) elicited any reporter gene activity in yeast suggesting that their transcriptional activation domains were either not present, not folded properly, or incompatible with the yeast system. The C-terminal 42 aa residues of GmHSF34 showed a strong negative effect on transcriptional activity of the GAL4 activation domain when both were adjacent in the same protein.

As a whole, the functional analysis of human and soybean HSFs in yeast cells indicated that these types of heterologous expression studies have limited usefulness which may be partly attributed to differences in regulation between yeast, plant and human cells. For hHSF1, these differences were more apparent when studying the mechanism of basal repression and negative control by the NR domain. However, the yeast expression system was useful in special situations, such as the mapping of transcriptional activation domains and in the screening of point mutations with CTA1. In the case of soybean HSFs, very little was learned from the yeast expression studies because too little was known regarding their function in plants. It is clear that in order to understand the mechanisms of HSF regulation, a more direct approach is potentially more informative. For this reason, protein-protein interaction studies involving transcriptional regulatory domains of hHSF1 and GTFs were conducted both *in vitro* and *in vivo* in order to identify potential targets of contact in the PIC.

The results of protein interaction studies suggest that both hTBP and hTFIIB are major targets for hHSF1 in the activation of transcription. The evidence supporting involvement of hTBP is based on *in vitro* mapping studies indicating that it has affinity for full length hHSF1, AD1/HR-C and CTA1/AD2 domains. These interactions were demonstrated using both *E. coli*- and HeLa-expressed T7-tagged hTBP. The link to biological relevance was made by showing that a hTBP mutant was able to squelch activity of a GAL4-DBD-CTA1-Plus effector in HeLa cells, and by showing that this inhibition did not occur when a second hTBP mutant that was lacking binding site for CTA1-Plus (the N-terminal core repeat) was co-expressed. An additional proof was obtained by the correlation between the ability of hTBP to bind wild type and mutated CTA1 (MA-1) and transcriptional activities in HeLa cells (Fig. 14 and Table 1). The final confirmation was achieved by showing a strong interaction between CTA1-Plus and the endogenous TFIID complex (Fig. 15).

The evidence regarding the involvement of hTFIIB is not as extensive, but still indicates that hTFIIB is also a major target for hHSF1 contact. *In vitro* hTFIIB has relatively strong affinity for HR-C/AD1 and weak affinity for CTA1/AD2 (Fig. 19). Evidence demonstrating a potential for *in vivo* interactions between hHSF1 and hTFIIB is derived from the strong squelch of GAL4-DBD-CTA1-Plus activity obtained when two dysfunctional mutants of hTFIIB ($hTFIIB_{1-108}$ and $hTFIIB_{109-207}$) are co-expressed in HeLa transient assays (Fig. 20).

PC4, TAF32 and TAF55 seem not to be the direct functional targets of hHSF1 (Fig. 21 to 23). In view of the high degree in conservation in TBPs and TFIIBs between divergent organisms, it is not surprising that the transcriptional activation domains of hHSF1 can function as strong activators in a variety of heterologous systems.

The ability of the negative regulation domain (NR) of hHSF1 to bind TFIID and repress CTA1-Plus-mediated reporter gene activity (Fig. 25 and 26) suggests that the interaction between the NR and TFIID results in the formation of a dysfunctional complex that is transcriptionally incompetent. A model incorporating the formation of a dysfunctional complex between the NR and TFIID under both hs and non-hs conditions attributes the interplay among the NR and activation domains of hHSF1 with TFIID as the basis for control of the final step in heat induced activation of gene expression.

These studies in yeast, HeLa and *in vitro* have mapped the transcriptional activation domains of hHSF1 and hHSF2 to their C-termini and have identified two GTFs that have the potential to be the major targets of interaction in the PIC. Contact with the PIC presumably represents the final step in the signal transduction pathway responsible for heat stress-induced expression of hs genes. One of the unresolved questions concerns the process whereby the C-terminal domains of hHSF1 are activated upon heat stress so that contact is made with hTBP and hTFIIB. At present, masking models for

basal repression of the C-terminus are favored, but the nature of this unmasking, whether by folding or by interaction with other proteins, remains a challenging area of research.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Docter of Philosophy.

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